

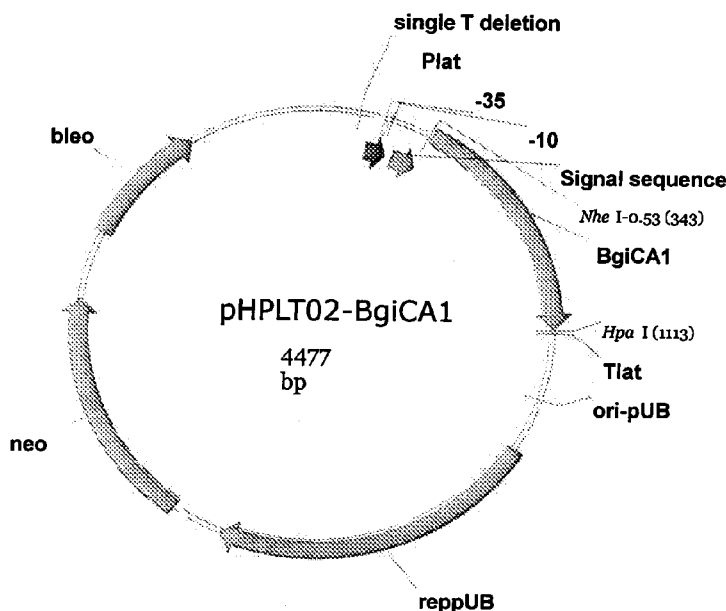
(43) International Publication Date
15 November 2012 (15.11.2012)

- (51) **International Patent Classification:**
- | | | | | |
|------------------------------|-----------------------------|-------------------|-------------------------------|----|
| <i>C12N 9/88</i> (2006.01) | <i>C12N 15/63</i> (2006.01) | PCT/CN2011/084366 | 21 December 2011 (21.12.2011) | CN |
| <i>C07K 14/195</i> (2006.01) | <i>C12N 15/52</i> (2006.01) | PCT/CN2011/084384 | 21 December 2011 (21.12.2011) | CN |
| <i>C07K 14/37</i> (2006.01) | <i>C12P 5/02</i> (2006.01) | 61/578,408 | 21 December 2011 (21.12.2011) | US |
| <i>C07K 17/02</i> (2006.01) | | 61/578,416 | 21 December 2011 (21.12.2011) | US |
| | | 61/578,421 | 21 December 2011 (21.12.2011) | US |
| | | 61/578,429 | 21 December 2011 (21.12.2011) | US |
- (21) **International Application Number:** PCT/US2012/036932
- (22) **International Filing Date:** 8 May 2012 (08.05.2012)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**
- | | | |
|-------------------|-------------------------------|----|
| PCT/CN2011/073886 | 10 May 2011 (10.05.2011) | CN |
| PCT/CN2011/073876 | 10 May 2011 (10.05.2011) | CN |
| PCT/CN2011/073873 | 10 May 2011 (10.05.2011) | CN |
| PCT/CN2011/073871 | 10 May 2011 (10.05.2011) | CN |
| PCT/CN2011/084371 | 21 December 2011 (21.12.2011) | CN |
| PCT/CN2011/084363 | 21 December 2011 (21.12.2011) | CN |
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[Continued on next page]

(54) **Title:** THERMOSTABLE CARBONIC ANHYDRASES AND METHODS OF USE THEREOF

(57) **Abstract:** The present compositions and methods relate to a thermostable carbonic anhydrases, polynucleotides encoding the carbonic anhydrase, and methods of make and/or use thereof. Formulations containing the carbonic anhydrase are suitable for use in extracting carbon dioxide.

**Figure 1**



(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

THERMOSTABLE CARBONIC ANHYDRASES AND METHODS OF USE THEREOF**CROSS-REFERENCE**

[001] This application claims the benefit of Chinese PCT application number PCT/CN2011/073886 filed May 10, 2011; Chinese PCT application number PCT/CN2011/073876 filed May 10, 2011; Chinese PCT application number PCT/CN2011/073873 filed May 10, 2011; Chinese PCT application number PCT/CN2011/073871 filed May 10, 2011; Chinese PCT application number PCT/CN2011/084371 filed December 21, 2011; Chinese PCT application number PCT/CN2011/084366 filed December 21, 2011; Chinese PCT application number PCT/CN2011/084363 filed December 21, 2011; Chinese PCT application number PCT/CN2011/084384 filed December 21, 2011; US provisional application number 61/578408 filed on December 21, 2011; US provisional application number 61/578429 filed on December 21, 2011; US provisional application number 61/578416 filed on December 21, 2011; and US provisional application number 61/578421 filed on December 21, 2011; the filing date of and each of these provisional applications are hereby expressly incorporated by reference in their entirety.

BACKGROUND

[002] Much of the world's electrical power is generated from the combustion of fossil-fuels such as coal, natural gas and fuel oil. Carbon dioxide (CO₂) is a by-product of combustion and is the principal greenhouse gas. As such, CO₂ emissions are believed to be a major contributor to the phenomenon of global warming, which could lead to drastic climate changes (e.g., violent weather patterns, changes in precipitation, rising ocean levels, and increases in temperature). Additionally, because CO₂ is an acidic gas, it forms the corrosive product carbonic acid in the presence of water.

[003] Several approaches for extraction CO₂ have been suggested, including transformation by algae, sequestration in oceans, storage in depleted oil and natural gas wells, dissolution in deep water tables, and transformation into carbonates. However, many of these approaches have significant drawbacks, and are often economically or ecologically unviable.

[004] Carbonic anhydrases (EC 4.2.1.1; and CAS No. 9001-03-0) catalyze the inter-conversion between carbon dioxide and bicarbonate [CO₂ + H₂O ↔ HCO₃⁻ + H⁺]. The active site of most carbonic anhydrases contains a zinc ion, therefore; these enzymes are also classified as metalloenzymes. Carbonic anhydrases may serve as biological catalysts to accelerate the capture of CO₂. Naturally occurring carbonic anhydrases typically function at low temperatures and are poorly suited to extraction of CO₂ from hot combustion gases. Accordingly, the need exists for carbonic anhydrases that can effectively hydrate CO₂ under inhospitable conditions (e.g., elevated temperatures, alkaline pH, and/or in the presence of high concentrations of bicarbonate).

SUMMARY

[005] The present disclosure provides compositions and methods that relate to recombinant carbonic anhydrases from *Bacillus gibsonii*, *Promicromonospora vindobonensis*, *Aspergillus terreus*, *Streptomyces pristinaespiralis*, *Bacillus agaradhaerens*, *Vibrio sp. AND4*, *Vibrio sp. Ex25*,
5 polynucleotides encoding the carbonic anhydrases, and methods of make and/or use thereof. Formulations containing the recombinant carbonic anhydrases are suitable for use in extracting carbon dioxide.

[006] In some embodiments, the present compositions and methods relate to a recombinant carbonic anhydrase 1 from *Bacillus gibsonii* (Bgi CA1). In some embodiments, the present compositions
10 and methods relate to a recombinant carbonic anhydrase 1 from *Promicromonospora vindobonensis* (Pvi CA1). In some embodiments, the present compositions and methods relate to a recombinant carbonic anhydrase 1 cloned from *Aspergillus terreus* (Ate CA1). In some embodiments, the present compositions and methods relate to a recombinant carbonic anhydrase 1 from *Streptomyces pristinaespiralis* (Spr CA1). In some embodiments, the present compositions and methods relate to a recombinant carbonic
15 anhydrase 1 from *Bacillus agaradhaerens* (Bag CA1). In some embodiments, the present compositions and methods relate to a recombinant carbonic anhydrase 1 from *Vibrio sp. AND4* (Vsp CA1). In some embodiments, the present compositions and methods relate to a recombinant carbonic anhydrase I from *Vibrio sp. Ex25* (VspE CA1). In some embodiments, the present compositions and methods relate to a recombinant carbonic anhydrase II from *Vibrio sp. Ex25* (VspE CA2). Formulations containing one or
20 more of the recombinant carbonic anhydrases are suitable for use in extracting carbon dioxide from a carbon dioxide-containing medium, such as combustion exhaust and flue gas streams.

[007] In one aspect, recombinant polypeptides are provided, comprising an amino acid sequence that is at least 55% (e.g., 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 28, SEQ ID NO: 43, SEQ ID
25 NO: 53, SEQ ID NO: 64, SEQ ID NO: 80, SEQ ID NO: 130, or SEQ ID NO: 136, wherein the polypeptide has carbonic anhydrase activity. In some embodiments, the recombinant polypeptide is contained in a basic, high ionic strength solution.

[008] In some embodiments, the invention provides a recombinant polypeptide comprising an amino acid sequence, where the amino acid sequence has carbonic anhydrase activity, where the amino
30 acid sequence has a higher thermostability and/or melting temperature at a higher ionic strength, and where the amino acid sequence is at least 70 % identical to the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 28, SEQ ID NO: 43, SEQ ID NO: 53, SEQ ID NO: 64, SEQ ID NO: 80, SEQ ID NO: 130, or SEQ ID NO: 136.

[009] In some embodiments, the invention provides a recombinant polypeptide comprising an
35 amino acid sequence, where the amino acid sequence has carbonic anhydrase activity greater than 25%

of the carbonic anhydrase activity when incubated for 30 minutes or longer at a pH of from about 8 to about 10.5, and where the amino acid sequence is at least 70 % identical to the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 28, SEQ ID NO: 43, SEQ ID NO: 53, SEQ ID NO: 64, SEQ ID NO: 80, SEQ ID NO: 130, or SEQ ID NO: 136.

- 5 **[0010]** In one aspect, recombinant polypeptides are provided, comprising an amino acid sequence that is at least 55% (e.g., 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to the amino acid sequence of SEQ ID NO: 3, wherein the polypeptide has carbonic anhydrase activity. In some embodiments, the recombinant polypeptide is contained in a basic, high ionic strength solution. In some embodiments, the polypeptide retains greater than 70% (e.g., greater than 70%, greater than 75%, greater than 80%, greater than 85%, greater than 90%, greater than 95%, greater than 96%, greater than 97%, greater than 98%, greater than 99%, or 100%) of the carbonic anhydrase activity when incubated for 5 or more hours at a pH of from 8.5 to 10.5, which, in some embodiments, takes place at a temperature of from 25°C to 40°C. In some embodiments, the polypeptide retains at least 90% (e.g., at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100%) of the carbonic anhydrase activity when incubated for about 24 hours at a temperature of about 25°C, which, in some embodiments, is at a pH of from 8.5 to 10.5. In some embodiments, the polypeptide retains at least 90% (e.g., at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100%) of the carbonic anhydrase activity when incubated for about 24 hours at a temperature of about 40°C, which, in some embodiments, is at a pH of from 8.5 to 10.5. In some embodiments, the polypeptide retains at least 50% (e.g., at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for about 3 hours at a temperature of about 50°C, which, in some embodiments, is at a pH of from 8.5 to 9.5. In some embodiments, the polypeptide retains at least 25% (e.g., at least 25%, at least 35%, at least 45%, at least 55%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for about 3 hours at a temperature of about 50°C, which, in some embodiments, is at a pH of 10.5. In some embodiments, the polypeptide retains at least 50% (e.g., at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for 1 to 3 hours at a temperature of from 20°C to 50°C under high ionic strength conditions. In some embodiments, the high ionic strength conditions comprise 1 M bicarbonate. In some embodiments, the polypeptide has a melting temperature that is elevated in the presence of 1-2 M bicarbonate.
- 35 **[0011]** In one aspect, recombinant polypeptides are provided, comprising an amino acid sequence that is at least 55% (e.g., 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or

100%) identical to the amino acid sequence of SEQ ID NO: 28, wherein the polypeptide has carbonic anhydrase activity. In some embodiments, the recombinant polypeptide is contained in a basic, high ionic strength solution. In some embodiments, the polypeptide retains greater than 70% (e.g., greater than 70%, greater than 75%, greater than 80%, greater than 85%, greater than 90%, greater than 95%, greater than 96%, greater than 97%, greater than 98%, greater than 99%, or 100%) of the carbonic anhydrase activity when incubated for 5 or more hours at a pH of from 8.5 to 9.5, which, in some embodiments, takes place at a temperature of from 25°C to 40°C. In some embodiments, the polypeptide retains at least 80% (e.g., at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100%) of the carbonic anhydrase activity when incubated for about 24 hours at a temperature of about 25°C, which, in some embodiments, is at a pH of from 8.5 to 9.5. In some embodiments, the polypeptide retains at least 80% (e.g., at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100%) of the carbonic anhydrase activity when incubated for about 24 hours at a temperature of about 40°C, which, in some embodiments, is at a pH of from 8.5 to 9.5. In some embodiments, the polypeptide retains at least 40% (e.g., at least 40%, at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for about 3 hours at a temperature of about 50°C, which, in some embodiments, is at a pH of from 8.5 to 9.5. In some embodiments, the polypeptide retains at least 25% (e.g., at least 25%, at least 35%, at least 45%, at least 55%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for about 3 hours at a temperature of about 40°C, which, in some embodiments, is at a pH of 10.5. In some embodiments, the polypeptide retains at least 50% (e.g., at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for 1 to 3 hours at a temperature of from 20°C to 50°C under high ionic strength conditions. In some embodiments, the high ionic strength conditions comprise 1 M bicarbonate. In some embodiments, the polypeptide has a melting temperature that is elevated in the presence of 1-2 M bicarbonate.

[0012] In one aspect, recombinant polypeptides are provided, comprising an amino acid sequence that is at least 55% (e.g., 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to the amino acid sequence of SEQ ID NO: 43, wherein the polypeptide has carbonic anhydrase activity. In some embodiments, the recombinant polypeptide is contained in a basic, high ionic strength solution. In some embodiments, the polypeptide retains greater than 70% (e.g., greater than 70%, greater than 75%, greater than 80%, greater than 85%, greater than 90%, greater than 95%, greater than 96%, greater than 97%, greater than 98%, greater than 99%, or 100%) of the carbonic anhydrase activity when incubated for 5 or more hours at a pH of from 8.0 to 11 (e.g., from 8.0 to 10.5,

from 8.5 to 10.5, from 8.5 to 11, from 8.5 to 10, from 8.0 to 10, from 9.0 to 11, etc), which, in some embodiments, takes place at a temperature of from 25°C to 40°C. In some embodiments, the polypeptide retains at least 90% (e.g., at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100%) of the carbonic anhydrase activity when incubated for about 24 hours at a temperature of about 25°C, which, in some embodiments, is at a pH of from 8.5 to 10.5. In some embodiments, the polypeptide retains at least 80% (e.g., at least 80%, at least 82%, at least 84%, at least 86%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100%) of the carbonic anhydrase activity when incubated for about 24 hours at a temperature of about 40°C, which, in some embodiments, is at a pH of from 8.5 to 10.5. In some embodiments, the polypeptide retains at least 40% (e.g., at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for about 3 hours at a temperature of about 50°C, which, in some embodiments, is at a pH of from 8.5 to 10 (e.g., 8.5 to 9.5, 8.5 to 10, 9 to 10, etc). In some embodiments, the polypeptide retains at least 25% (e.g., at least 25%, at least 35%, at least 45%, at least 55%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for about 3 hours at a temperature of about 50°C, which, in some embodiments, is at a pH of 10.5. In some embodiments, the polypeptide retains at least 50% (e.g., at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for 1 to 3 hours at a temperature of from 20°C to 50°C under high ionic strength conditions. In some embodiments, the high ionic strength conditions comprise 1 M bicarbonate. In some embodiments, the polypeptide has a melting temperature that is elevated in the presence of 1-2 M bicarbonate. In related embodiments, the polypeptide has increased thermostability at higher ionic strength conditions. For example, the polypeptide has a higher melting temperature at a higher ionic strength condition (e.g., at 1 M bicarbonate) as compared to at a lower ionic strength condition (e.g., at 0.8, 0.6, 0.4, 0.2, or 0.1 M bicarbonate).

[0013] In one aspect, recombinant polypeptides are provided, comprising an amino acid sequence that is at least 55% (e.g., 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to the amino acid sequence of SEQ ID NO: 53, wherein the polypeptide has carbonic anhydrase activity. In some embodiments, the recombinant polypeptide is contained in a basic, high ionic strength solution. In some embodiments, the polypeptide retains greater than 50% (e.g., greater than 50%, greater than 60%, greater than 70%, greater than 75%, greater than 80%, greater than 85%, greater than 90%, greater than 95%, greater than 96%, greater than 97%, greater than 98%, greater than 99%, or 100%) of the carbonic anhydrase activity when incubated for 5 or more hours at a pH of from 8 to 10 (e.g., 8 to 9.5, 8.5 to 9.5, 8.5 to 10, 8 to 10, etc), which, in some embodiments, takes place at a

temperature of from 25°C to 40°C. In some embodiments, the polypeptide retains at least 70% (e.g., at least 70%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100%) of the carbonic anhydrase activity when incubated for about 24 hours at a temperature of about 25°C, which, in some embodiments, is at a pH of from 8 to 11 (e.g., 8 to 10.5, 8.5 to 10.5, 8.5 to 11, 8.5 to 10, etc). In some embodiments, the polypeptide retains at least 50% (e.g., at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100%) of the carbonic anhydrase activity when incubated for about 24 hours at a temperature of about 40°C, which, in some embodiments, is at a pH of from 8.5 to 10.5 (e.g., 8.5 to 10, 8.5 to 10.5, 8.5 to 9.5, 8 to 10, 8 to 9.5, etc). In some embodiments, the polypeptide retains at least 30% (e.g., at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for about 2 hours at a temperature of about 50°C, which, in some embodiments, is at a pH of from 8 to 11 (e.g., 8 to 10.5, 8.5 to 10.5, 8.5 to 11, 8.5 to 10, 9 to 10.5, etc). In some embodiments, the polypeptide retains at least 15% (e.g., at least 15%, at least 20%, at least 25%, at least 35%, at least 45%, at least 55%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for about 2 hours at a temperature of about 50°C, which, in some embodiments, is at a pH of 8, 8.5, 9.0, 9.5, 10, 10.5, or 11. In some embodiments, the polypeptide retains at least 50% (e.g., at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for 1 to 3 hours at a temperature of from 20°C to 50°C under high ionic strength conditions. In some embodiments, the high ionic strength conditions comprise 1 M bicarbonate. In some embodiments, the polypeptide has a melting temperature that is elevated in the presence of 1-2 M bicarbonate.

[0014] In one aspect, recombinant polypeptides are provided, comprising an amino acid sequence that is at least 55% (e.g., 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to the amino acid sequence SEQ ID NO: 64, wherein the polypeptide has carbonic anhydrase activity. In some embodiments, the recombinant polypeptide is contained in a basic, high ionic strength solution. In some embodiments, the polypeptide retains greater than 70% (e.g., greater than 70%, greater than 75%, greater than 80%, greater than 85%, greater than 90%, greater than 95%, greater than 96%, greater than 97%, greater than 98%, greater than 99%, or 100%) of the carbonic anhydrase activity when incubated for 5 or more hours at a pH of from about 8.0 to about 11 (e.g., about 8.5 to about 10.5, about 8.5 to about 9.5, about 9.5 to about 10.5, about 8.5 to about 10, about 9 to about 10.5, etc), which, in some embodiments, takes place at a temperature of from about 20°C to about 55°C (e.g., about 25°C to about 50°C, about 30°C to about 45°C, about 35°C to 40°C, etc). In some

embodiments, the polypeptide retains at least 90% (e.g., at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100%) of the carbonic anhydrase activity when incubated for about 3, about 5, about 10, about 24 hours or longer at a temperature of about 25°C, which, in some embodiments, is at a pH of from 8.0 to 11 (e.g., 8.5 to 10.5, 8.5 to 9.5, 9.5 to 10.5, 8.5 to 10, 9 to 10, 9 to 10.5, etc). In some embodiments, the polypeptide retains at least 90% (e.g., at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100%) of the carbonic anhydrase activity when incubated for about 3, about 5, about 10, about 24 hours at a temperature of about 40°C, which, in some embodiments, is at a pH of from 8.0 to 11 (e.g., 8.5 to 10.5, 8.5 to 9.5, 9.5 to 10.5, 8.5 to 10, 9 to 10, 9 to 10.5, etc). In some embodiments, the polypeptide retains at least 50% (e.g., at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for about 3 hours at a temperature of about 50°C, which, in some embodiments, is at a pH of from about 8 to about 10 (e.g., about 8.5 to about 9.5, etc). In some embodiments, the polypeptide retains at least 25% (e.g., at least 25%, at least 35%, at least 45%, at least 55%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for about 3 hours at a temperature of about 50°C, which, in some embodiments, is at a pH of about 9.5 to 11 (e.g., about 10 to about 10.5, etc). In some embodiments, the polypeptide retains at least 50% (e.g., at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for as long as 10 hours (e.g., for 1 to 3 hours, for 1 to 5 hours, for 1 to 7 hours, for 1 to 9 hours, etc) at a temperature of from about 20°C to about 55°C (e.g., about 25°C to about 50°C, about 20°C to about 50°C, about 30°C to about 50°C, etc) under high ionic strength conditions. In some embodiments, the high ionic strength conditions comprise 1 M bicarbonate. In some embodiments, the polypeptide has a melting temperature that is elevated in the presence of 1 to 2 M bicarbonate.

[0015] In one aspect, recombinant polypeptides are provided, comprising an amino acid sequence that is at least 55% (e.g., 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to the amino acid sequence of SEQ ID NO: 80, wherein the polypeptide has carbonic anhydrase activity. In some embodiments, the recombinant polypeptide is contained in a basic, high ionic strength solution. In some embodiments, the polypeptide retains greater than 50% (e.g., greater than 50%, greater than 55%, greater than 60%, greater than 65%, greater than 70%, greater than 75%, greater than 80%, greater than 85%, greater than 90%, greater than 95%, greater than 96%, greater than 97%, greater than 98%, greater than 99%, or 100%) of the carbonic anhydrase activity when incubated for 3 or more hours at a pH of from about 8 to about 11 (e.g., from about 8 to about 10.5, from about 8.5 to about 9.5, from about 8.2 to about 10.6, from about 8.5 to about 10.5, from about 8.4 to about 10.2, from about 8.6 to about 10, or from 9.5 to about 10.5, etc), which, in some embodiments, takes place at a

temperature of from 20°C to 55°C (e.g., from 25°C to 50°C, 30°C to 45°C, 35°C to 40°C, etc). In some embodiments, the polypeptide retains at least 60% (e.g., at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for at least about 1 hour, at least about 5 hours, at least about 10 hours, at least about 24 hours, or longer at a temperature of about 25°C, which, in some embodiments, is at a pH of from about 8 to about 11 (e.g., about 8 to about 10.8, about 8.2 to about 10.6, about 8.5 to about 10.5, about 8.5 to about 9.5, about 9.5 to about 10.5, about 8.6 to about 10.2, about 8.8 to about 10, etc). In some embodiments, the polypeptide retains at least 50% (e.g., at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for longer than about 1 hour, about 3 hours, about 5 hours, about 10 hours, about 24 hours, or longer, at a temperature of about 40°C, which, in some embodiments, is at a pH of from about 8 to about 10 (e.g., about 8 to about 10.8, about 8.5 to about 9.5, about 9.5 to about 10.5, about 8.2 to about 10.6, about 8.5 to about 10.5, about 8.6 to about 10.2, about 8.8 to about 10, etc). In some embodiments, the polypeptide retains at least 50% (e.g., at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for about 1 hour, about 3 hours, about 5 hours, about 10 hours, or at least 24 hours, at a temperature of about 50°C, which, in some embodiments, is at a pH of about 8.5. In some embodiments, the polypeptide retains at least 20% (e.g., at least 20%, at least 25%, at least 35%, at least 45%, at least 55%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for about 1 hour, about 2 hours, or about 3 hours at a temperature of about 50°C, which, in some embodiments, is at a pH of about 9.5 to about 10.5. In some embodiments, the polypeptide retains at least 30% (e.g., at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for 1 to 3 hours at a temperature of about 20°C under high ionic strength conditions. In some embodiments, the polypeptide retains at least about 30% (e.g., at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65% or at least 70%) of the carbonic anhydrase activity when incubated for 10 minutes to 40 minutes, under high ionic strength conditions. In some embodiments, the high ionic strength conditions comprise 1 M bicarbonate. In some embodiments, the polypeptide has a melting temperature that is elevated in the presence of 1-2 M bicarbonate. In related embodiments, the polypeptide has an increased thermostability at a higher ionic strength. For example, the polypeptide can suitably have an increased melting temperature at a higher ionic strength

(e.g., at 1 M bicarbonate) than at a lower ionic strength (e.g., at 0.8 M bicarbonate, at 0.6 M bicarbonate, at 0.4 M bicarbonate, at 0.2 M bicarbonate, or at 0.1 M bicarbonate, etc).

[0016] In one aspect, recombinant polypeptides are provided, comprising an amino acid sequence that is at least 55% (e.g., 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to the amino acid sequence of SEQ ID NO:130, wherein the polypeptide has carbonic anhydrase activity. In some embodiments, the recombinant polypeptide is contained in a basic, high ionic strength solution. In some embodiments, the polypeptide retains greater than 50% (e.g., greater than 50%, greater than 55%, greater than 60%, greater than 65%, greater than 70%, greater than 75%, greater than 80%, greater than 85%, greater than 90%, greater than 95%, greater than 96%, greater than 97%, greater than 98%, greater than 99%, or 100%) of the carbonic anhydrase activity when incubated for 1 or more, 2 or more, 3 or more, 4 or more, or 5 or more hours at a pH of from about 8 to about 11 (e.g., about 8.5 to about 10.5, about 8.5 to about 9.5, about 9.5 to about 10.5, about 8 to about 10.5, etc), which, in some embodiments, takes place at a temperature of from about 20°C to about 55°C (e.g., about 25°C to about 50°C, about 25°C to about 40°C, about 40°C to about 55°C, etc). In some embodiments, the polypeptide retains at least 60% (e.g., at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for about 30 minutes or longer, 1 hour or longer, 2 hours or longer, 3 hours or longer, 5 hours or longer, 10 hours or longer, 15 hours or longer, or even 24 hours or longer, at a temperature of about 25°C, which, in some embodiments, is at a pH of from about 8 to about 11 (e.g., about 8.5 to about 10.5, about 9.5 to about 10.5, about 8.5 to about 9.5). In some embodiments, the polypeptide retains at least 70% (e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100%) of the carbonic anhydrase activity when incubated for about 30 minutes, about 1 hour, about 2 hours, about 3 hours, about 5 hours, about 10 hours, about 15 hours, or about 24 hours at a temperature of about 40°C, which, in some embodiments, is at a pH of from about 8 to about 11 (e.g., about 8.5 to about 10.5, about 8.5 to about 9.5, about 9.5 to about 10.5, about 8 to about 10.5, etc). In some embodiments, the polypeptide retains at least 30% (e.g., at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for about 30 minutes, about 1 hour, about 2 hours, about 3 hours, or longer at a temperature of about 50°C, which, in some embodiments, is at a pH of from about 8 to about 11 (e.g., about 8.5 to about 10.5, about 8.5 to about 9.5, about 9.5 to about 10.5, about 8 to about 10.5, etc). In some embodiments, the polypeptide retains at least 15% (e.g., at least 15%, at least 20%, at least 25%, at least 35%, at least 45%, at least 55%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for about 30 minutes, 1 hour, 2 hours, or 3 hours or longer, at a

temperature of about 50°C, which, in some embodiments, is at a pH of between about 8.5 to about 11 (e.g., about 8.5 to about 10.5, about 8.5 to about 9.5, about 9.5 to about 10.5, about 8 to about 10.5, or at individual pH values of about 8, about 8.5, about 9.0, about 9.5, about 10, about 10.5, or about 11). In some embodiments, the polypeptide retains at least 30% (e.g., at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for about 30 minutes, about 1 hour, about 2 hours, about 3 hours, or longer, at a temperature of from 20°C to 50°C under high ionic strength conditions. In some embodiments, the high ionic strength conditions comprise 1 M bicarbonate. In some embodiments, the polypeptide has a melting temperature that is elevated in the presence of 1-2 M bicarbonate. In related embodiments, the polypeptide has improved or higher thermostability at higher ionic strength conditions. For example, the polypeptide has a higher melting temperature at a higher ionic strength (e.g., 1 M bicarbonate) as compared to its melting temperature at a lower ionic strength (e.g., 0.8 M bicarbonate, 0.6 M bicarbonate, 0.4 M bicarbonate, 0.2 M bicarbonate, 0.1 M bicarbonate, etc).

[0017] In one aspect, recombinant polypeptides are provided, comprising an amino acid sequence that is at least 55% (e.g., 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to the amino acid sequence of EQ ID NO: 136, wherein the polypeptide has carbonic anhydrase activity. In some embodiments, the recombinant polypeptide is contained in a basic, high ionic strength solution. In some embodiments, the polypeptide retains greater than 25% (e.g., greater than 25%, greater than 30%, greater than 35%, greater than 40%, greater than 45%, greater than 50%, greater than 55%, greater than 60%, greater than 65%, greater than 70%, greater than 75%, greater than 80%, greater than 85%, greater than 90%, greater than 95%, or 100%) of the carbonic anhydrase activity when incubated for 30 minutes or longer, 1 hour, 2 hours, 3 hours, 5 hours, 10 hours, or longer at a pH of from about 8 to about 11 (e.g., about 8.5 to about 10.5, about 8.5 to about 9.5, about 9.5 to about 10.5, about 8 to about 10, etc), which, in some embodiments, takes place at a temperature of from 20°C to 55°C (e.g., 25°C to 40°C, 40°C to 55°C, 20°C to 50°C, etc). In some embodiments, the polypeptide retains at least 40% (e.g., at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for at least about 30 minutes, at least about 1 hour, at least about 2 hours, at least about 3 hours, at least about 5 hours, at least about 10 hours, at least about 24 hours, or longer at a temperature of about 25°C, which, in some embodiments, is at a pH of from about 8 to about 11 (e.g., about 8.5 to about 10.5, about 8.5 to about 9.5, about 9.5 to about 10.5, about 8 to about 10, etc). In some embodiments, the polypeptide retains at least 60% (e.g., at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100%) of the carbonic anhydrase activity when incubated for at least 30 minutes, at least 1 hour, at least 2 hours, at least 5 hours, at least 10 hours, or at least 24 hours, or longer at a temperature of about 40°C, which, in some embodiments, is

at a pH of from about 8 to about 11 (e.g., about 8.5 to about 10.5, about 8.5 to about 9.5, about 9.5 to about 10.5, about 8 to about 10, etc). In some embodiments, the polypeptide retains at least 25% (e.g., at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for about 30 minutes, about 1 hour, about 2 hours, about 3 hours, or longer, at a temperature of about 50°C, which, in some embodiments, is at a pH of from about 8 to about 11 (e.g., about 8.5 to about 9.5, about 8.5 to about 10.5, about 8 to about 10, about 9.5 to about 10.5, etc.). In some embodiments, the polypeptide retains at least 25% (e.g., at least 25%, at least 35%, at least 45%, at least 55%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for about 30 minutes, about 1 hour, about 2 hours, about 3 hours, or longer, at a temperature of about 50°C, which, in some embodiments, is at a pH of 10.5. In some embodiments, the polypeptide retains at least 40% (e.g., at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the carbonic anhydrase activity when incubated for 1 to 3 hours at a temperature of from 20°C to 50°C under high ionic strength conditions. In some embodiments, the high ionic strength conditions comprise 1 M bicarbonate. In some embodiments, the polypeptide has a melting temperature that is elevated in the presence of 1-2 M bicarbonate. In related embodiments, the polypeptide has an increased or higher thermostability at a higher ionic strength conditions. For example, the polypeptide has a higher melting temperature at a higher ionic strength (e.g., 1 M bicarbonate) as compared to at a lower ionic strength (e.g., 0.8 M bicarbonate, 0.6 M bicarbonate, 0.4 M bicarbonate, 0.2 M bicarbonate, or 0.1 M bicarbonate, etc).

[0018] In some embodiments, the polypeptide comprises an amino acid sequence that is at least 70% (e.g., at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) identical to the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 28, SEQ ID NO: 43, SEQ ID NO: 53, SEQ ID NO: 64, SEQ ID NO: 80, SEQ ID NO:130, or SEQ ID NO: 136, at least 80% (e.g., at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) identical to the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 28, SEQ ID NO: 43, SEQ ID NO: 53, SEQ ID NO: 64, SEQ ID NO: 80, SEQ ID NO:130, or SEQ ID NO: 136, at least 90% (e.g., at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) identical to the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 28, SEQ ID NO: 43, SEQ ID NO: 53, SEQ ID NO: 64, SEQ ID NO: 80, SEQ ID NO:130, or SEQ ID NO: 136, or at least 95% (e.g., at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) identical to the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 28, SEQ ID NO: 43, SEQ ID NO: 53, SEQ ID NO: 64, SEQ ID NO: 80, SEQ ID NO:130, or

SEQ ID NO: 136. Also provided are compositions comprising the recombinant polypeptide and a carbon dioxide-containing medium. In some embodiments, the recombinant polypeptide is immobilized. In some embodiments, the recombinant polypeptide is immobilized on a polymer, a membrane, a matrix, a micellar material, a wafer, a solid support, or a micro-particle.

5 **[0019]** In certain aspect, an isolated nucleic acid is provided, which encodes a recombinant polypeptide of any of the preceding paragraph. In some embodiments, the polypeptide further comprises a signal peptide sequence. In some embodiments, the signal peptide and the carbonic anhydrase polypeptide are derived from different microorganisms. In certain embodiments, the signal peptide sequence is selected from the group consisting of SEQ ID NOs: 10-15, 69-73, 81- 83, and 131-132. Also
10 provided is an expression vector comprising the isolated nucleic acid in operable combination with a regulatory sequence. Additionally, a host cell is provided comprising the expression vector. In some embodiments, the host cell is a bacterial cell or a fungal cell. In still further embodiments, a composition is provided, which comprises the host cell and a culture medium.

15 **[0020]** In certain aspect, methods of producing a carbonic anhydrase are provided, comprising: culturing a host cell of the preceding paragraph in a culture medium, under suitable conditions to produce the carbonic anhydrase. Also provided are compositions that comprise the carbonic anhydrase produced in accordance with the methods provided herein in supernatant of the culture medium.

20 **[0021]** In a further aspect, methods for extracting carbon dioxide from a carbon dioxide-containing medium are provided, comprising: contacting the carbon dioxide-containing medium with the polypeptide of one of the preceding paragraphs to yield a medium reduced in carbon dioxide. In some
embodiments, the carbon dioxide-containing medium is selected from the group consisting of a gas, a liquid, and a multi-phase mixture. In some embodiments, the carbon dioxide-containing medium is a gas selected from the group consisting of a flue gas, a raw natural gas, a syngas, a hydrogen gas, and a biogas. In some embodiments, the carbon dioxide-containing medium is a by-product of combustion or
25 fermentation.

[0022] In certain aspect, methods for regulating pH of a medium containing one or both of CO₂ and bicarbonate are provided, comprising: contacting the medium with the polypeptide of one of the preceding paragraphs to yield a medium with an altered pH.

30 **[0023]** Also provided herein is a carbonic anhydrase or a suitable variant thereof, which has been engineered to achieve improved thermostability, improved activity/stability at certain alkaline pH, and/or in the presence of a high concentration of bicarbonate. In some embodiments, the carbonic anhydrase is a recombinant carbonic anhydrase derived from a *Bacillus gibsonii* carbonic anhydrase.

35 **[0024]** In certain embodiments, the recombinant carbonic anhydrase comprises an amino acid sequence that is at least 55% identical to the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 28, SEQ ID NO: 43, SEQ ID NO: 53, SEQ ID NO: 64, SEQ ID NO: 80, SEQ ID NO:130, or SEQ ID NO:

136, or is one that is encoded by a polynucleotide sequence that is at least 50% (e.g., 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%) identical to SEQ ID NO: 1, SEQ ID NO: 26, SEQ ID NO: 41, SEQ ID NO: 51, SEQ ID NO: 62, SEQ ID NO: 77, SEQ ID NO: 127, or SEQ ID NO: 133 or is capable of hybridizing under intermediate, high, or maximum stringency to a complement of SEQ ID NO: 1, SEQ ID NO: 26, SEQ ID NO: 41, SEQ ID NO: 51, SEQ ID NO: 62, SEQ ID NO: 77, SEQ ID NO: 127, or SEQ ID NO: 133. In certain embodiments, the recombinant carbonic anhydrase is expressed and/or produced in a suitable host organism as described herein.

[0025] In some embodiments, the improved thermostability is manifested or evidenced by the retention of at least about 30% (e.g., at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or a higher percentage) of enzymatic activity when incubated under a temperature of between about 30°C and about 85°C (e.g., between about 30°C and about 80°C, between about 40°C and about 70°C, between about 45°C and about 65°C, between about 40°C and about 60°C). In some embodiments, the improved activity/stability at certain alkaline pH is manifest or evidenced by the retention of at least about 25% (e.g., at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more) activity under a pH that is between about 8.0 and about 10.0 (e.g., between about 8.0 and about 9.8, between about 8.2 and about 9.6, between about 8.4 and about 9.4, between about 8.6 and about 9.5, etc). In some embodiments, the improved activity/stability in the presence of a high concentration of bicarbonate is manifest or evidenced by the retention of at least about 25% (e.g., at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more) activity when the enzyme is incubated in the presence of about 0.1 M, 0.2 M, 0.5 M, 0.7 M, 0.9 M, 1.0 M, 1.2 M, 1.5 M, 2.0 M or a higher concentration of HCO_3^- for an extended period of time, for example, for at least 1 hour, 2 hours, 3 hours, 5 hours, 10 hours, 12 hours, 24 hours, several days, a week, several weeks, a month, or longer. In related embodiments, the polypeptide has increased melting temperature at higher ionic strength conditions. The polypeptide can thus be said to have improved or increased thermostability at higher ionic strength conditions. For example, the melting temperature of the polypeptide is higher at a higher ionic strength condition (e.g., at 1 M bicarbonate) as compared to at a lower ionic strength condition (e.g., at 0.8 M, 0.6 M, 0.4 M, 0.2 M, or 0.1 M bicarbonate). In certain embodiments, the recombinant carbonic anhydrase comprises an amino acid sequence that is at least 55% identical to the amino acid sequence of SEQ ID NO: 3.

[0026] In some embodiments, the improved thermostability is manifested or evidenced by the retention of at least about 30% (e.g., at least about 40%, at least about 50%, at least about 60%, at least

about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or a higher percentage) of enzymatic activity when incubated under a temperature of between about 20°C and about 75°C (e.g., between about 25°C and about 60°C, between about 30°C and about 50°C, between about 25°C and about 55°C, between about 35°C and about 65°C). In some embodiments, the improved activity/stability at certain alkaline pH is manifest or evidenced by the retention of at least about 25% (e.g., at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more) activity under a pH that is between about 8.0 and about 11.0 (e.g., between about 8.0 and about 9.8, between about 8.2 and about 9.6, between about 8.4 and about 9.4, between about 8.6 and about 9.5, between about 8.5 and about 10.8, between about 9.0 and about 10.5, etc). In some embodiments, the improved activity/stability in the presence of a high concentration of bicarbonate is manifest or evidenced by the retention of at least about 25% (e.g., at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more) activity when the enzyme is incubated in the presence of about 0.1 M, 0.2 M, 0.5 M, 0.7 M, 0.9 M, 1.0 M, 1.2 M, 1.5 M, 2.0 M or a higher concentration of HCO_3^- for an extended period of time, for example, for at least 1 hour, 2 hours, 3 hours, 5 hours, 10 hours, 12 hours, 24 hours, several days, a week, several weeks, a month, or longer. In certain related embodiments, an improved and/or increased thermostability is observed as the ionic strength increases. For example, the melting temperature of the polypeptide is higher at a higher ionic strength (e.g., at 1 M bicarbonate) as compared to at a lower ionic strength (e.g., at 0.8, 0.6, 0.4, 0.2, or 0.1 M bicarbonate). In certain embodiments, the recombinant carbonic anhydrase comprises an amino acid sequence that is at least 55% identical to the amino acid sequence of SEQ ID NO: 28.

[0027] In some embodiments, the improved thermostability is manifested or evidenced by the retention of at least about 30% (e.g., at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or a higher percentage) of enzymatic activity when incubated under a temperature of between about 30°C and about 85°C (e.g., between about 30°C and about 80°C, between about 40°C and about 70°C, between about 45°C and about 65°C, between about 40°C and about 60°C). In some embodiments, the improved activity/stability at certain alkaline pH is manifest or evidenced by the retention of at least about 25% (e.g., at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more) activity under a pH that is between about 8.0 and about 11.0 (e.g., between about 8.0 and about 10.5, between about 8.5 and about 11, between about 8.5 and about 10.5, between about 8.5 and about 10,

between about 8.0 and about 9.5, between about 8.2 and about 10, between about 8.6 and about 10.8, etc). In some embodiments, the improved activity/stability in the presence of a high concentration of bicarbonate is manifest or evidenced by the retention of at least about 25% (e.g., at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more) activity when the enzyme is incubated in the presence of about 0.1 M, 0.2 M, 0.5 M, 0.7 M, 0.9 M, 1.0 M, 1.2 M, 1.5 M, 2.0 M or a higher concentration of HCO_3^- for an extended period of time, for example, for at least 1 hour, 2 hours, 3 hours, 5 hours, 10 hours, 12 hours, 24 hours, several days, a week, several weeks, a month, or longer. In related embodiments, the polypeptide provided herein has an improved thermostability under higher ionic strength conditions. For example, the polypeptide can suitably have a higher melting temperature at a higher ionic strength condition (e.g., at 1 M bicarbonate) as compared to at a lower ionic strength condition (e.g., at 0.8, 0.6, 0.4, 0.2, or 0.1 M bicarbonate). In certain embodiments, the recombinant carbonic anhydrase comprises an amino acid sequence that is at least 55% identical to the amino acid sequence of SEQ ID NO: 43.

[0028] In some embodiments, the improved thermostability is manifested or evidenced by the retention of at least about 30% (e.g., at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or a higher percentage) of enzymatic activity when incubated under a temperature of between about 30°C and about 85°C (e.g., between about 30°C and about 80°C, between about 40°C and about 70°C, between about 45°C and about 65°C, between about 40°C and about 60°C). In some embodiments, the improved activity/stability at certain alkaline pH is manifest or evidenced by the retention of at least about 25% (e.g., at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more) activity under a pH that is between about 8.0 and about 11.0 (e.g., between about 8.0 and about 11, between about 8 and about 10.5, between about 8.5 and about 10, between about 8.5 and about 9.8, between about 8.2 and about 9.6, between about 8.4 and about 9.4, between about 8.6 and about 9.5, etc). In some embodiments, the improved activity/stability in the presence of a high concentration of bicarbonate is manifest or evidenced by the retention of at least about 25% (e.g., at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more) activity when the enzyme is incubated in the presence of about 0.1 M, 0.2 M, 0.5 M, 0.7 M, 0.9 M, 1.0 M, 1.2 M, 1.5 M, 2.0 M or a higher concentration of HCO_3^- for an extended period of time, for example, for at least 1 hour, 2 hours, 3 hours, 5 hours, 10 hours, 12 hours, 24 hours, several days, a week, several weeks, a month, or longer. In

certain embodiments, the recombinant carbonic anhydrase comprises an amino acid sequence that is at least 55% identical to the amino acid sequence of SEQ ID NO: 53.

[0029] In some embodiments, the improved thermostability is manifested or evidenced by the retention of at least about 30% (e.g., at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or a higher percentage) of enzymatic activity when incubated under a temperature of between about 20°C and about 85°C (e.g., between about 25°C and about 80°C, between about 30°C and about 70°C, between about 35°C and about 65°C, between about 40°C and about 60°C, etc). In some embodiments, the improved activity/stability at certain alkaline pH is manifest or evidenced by the retention of at least about 25% (e.g., at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more) activity under a pH that is between about 8.0 and about 11 (e.g., between about 8.0 and about 10.5, between about 8.5 to about 9.5, between about 8.2 and about 10.2, between about 8.4 and about 10, between about 8.6 and about 9.8, between about 8.5 to about 10.5, between about 9.5 to about 10.5, etc.). In some embodiments, the improved activity/stability in the presence of a high concentration of bicarbonate is manifest or evidenced by the retention of at least about 25% (e.g., at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more) activity when the enzyme is incubated in the presence of about 0.1 M, 0.2 M, 0.5 M, 0.7 M, 0.9 M, 1.0 M, 1.2 M, 1.5 M, 2.0 M or a higher concentration of HCO_3^- for an extended period of time, for example, for at least 1 hour, 2 hours, 3 hours, 5 hours, 10 hours, 12 hours, 24 hours, several days, a week, several weeks, a month, or longer. In related embodiments, the carbonic anhydrase enzyme has higher or increased thermostability and/or melting temperature under higher ionic strength conditions. For example, a carbonic anhydrase polypeptide suitably has a higher melting temperature at a higher concentration of bicarbonate (e.g., at 1 M bicarbonate) as compared to its melting temperature at a lower concentration of bicarbonate (e.g., at 0.8, 0.6, 0.4, 0.2, or 0.1 M bicarbonate). In certain embodiments, the recombinant carbonic anhydrase comprises an amino acid sequence that is at least 55% identical to the amino acid sequence of SEQ ID NO: 64.

[0030] In some embodiments, the improved thermostability is manifested or evidenced by the retention of at least about 25% (e.g., at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or a higher percentage) of enzymatic activity when incubated under a temperature of between about 20°C and about 55°C (e.g., between about 20°C and about 50°C, between about 25°C and about 50°C, between about 25°C and about 40°C, between about 40°C and about 55°C, etc.). In some embodiments, the improved activity/stability at certain

alkaline pH is manifest or evidenced by the retention of at least about 20% (e.g., at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more) activity under a pH that is about 8.0 and about 11 (e.g., about 8.0 and about 10.8, about 8.2 and about 10.6, about 8.5 and about 10.5, about 8.2 and about 10, about 8.4 and about 9.8, about 8.5 to about 9.5, about 9.5 to about 10.5, etc), for a time period of at least about 0.5, about 1.0, about 2.0, about 3.0, about 4.0, or about 5.0 hours, or longer. In some embodiments, the improved activity/stability in the presence of a high concentration of bicarbonate is manifest or evidenced by the retention of at least about 20% (e.g., at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more) activity when the enzyme is incubated in the presence of about 0.1 M, 0.2 M, 0.5 M, 0.7 M, 0.9 M, 1.0 M, 1.2 M, 1.5 M, 2.0 M or a higher concentration of HCO_3^- for an extended period of time, for example, for at least 1 hour, 2 hours, 3 hours, 5 hours, 10 hours, 12 hours, 24 hours, several days, a week, several weeks, a month, or longer. In related embodiments, the carbonic anhydrase polypeptide has an increased thermostability at a higher ionic strength. For example, the carbonic anhydrase polypeptide can suitably have a higher melting temperature at a higher ionic strength condition (e.g., at 1 M bicarbonate) as compared to at a lower ionic strength condition (e.g., at 0.8 M bicarbonate, at 0.6 M bicarbonate, at 0.4 M bicarbonate, at 0.2 M bicarbonate, or at 0.1 M bicarbonate, etc). In certain embodiments, the recombinant carbonic anhydrase comprises an amino acid sequence that is at least 55% identical to the amino acid sequence of SEQ ID NO: 80.

[0031] In some embodiments, the improved thermostability is manifested or evidenced by the retention of at least about 25% (e.g., at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or a higher percentage) of enzymatic activity when incubated under a temperature of between about 20°C and about 55°C (e.g., between about 20°C and about 50°C, between about 25°C and about 55°C, between about 25°C and about 40°C, between about 30°C and about 50°C). In some embodiments, the improved activity/stability at certain alkaline pH is manifest or evidenced by the retention of at least about 25% (e.g., at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more) activity under a pH that is between about 8.0 and about 11 (e.g., between about 8.5 and about 10.5, between about 8.5 and about 9.5, between about 9.5 and about 10.5, between about 8 and about 10, etc). In some embodiments, the improved activity/stability in the presence of a high concentration of bicarbonate is manifest or evidenced by the retention of at least about 25% (e.g., at least about 25%, at least about 30%, at least about 35%, at least

about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more) activity when the enzyme is incubated in the presence of about 0.1 M, 0.2 M, 0.5 M, 0.7 M, 0.9 M, 1.0 M, 1.2 M, 1.5 M, 2.0 M or a higher concentration of HCO_3^- for an extended period of time, for example, for at least 30 minutes, at least 1 hour, 2 hours, 3 hours, 5 hours, 10 hours, 12 hours, 24 hours, several days, a week, several weeks, a month, or longer. In related embodiments, the carbonic anhydrase polypeptide suitably has increased or improved thermostability under higher ionic strength conditions. For example, the polypeptide can suitably have a higher melting temperature at a higher ionic strength (e.g., at 1 M bicarbonate) than at a lower ionic strength (e.g., at 0.8 M bicarbonate, at 0.6 M bicarbonate, at 0.4 M bicarbonate, at 0.2 M bicarbonate, or at 0.1 M bicarbonate, etc). In certain embodiments, the recombinant carbonic anhydrase comprises an amino acid sequence that is at least 55% identical to the amino acid sequence of SEQ ID NO: 130.

[0032] In some embodiments, the improved thermostability is manifested or evidenced by the retention of at least about 25% (e.g., at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or a higher percentage) of enzymatic activity when incubated under a temperature of between about 2⁰⁰C and about 8⁵⁰C (e.g., between about 2⁰⁰C and about 8⁰⁰C, between about 2⁰⁰C and about 7⁰⁰C, between about 3⁰⁰C and about 6⁵⁰C, between about 4⁰⁰C and about 6⁰⁰C). In some embodiments, the improved activity/stability at certain alkaline pH is manifest or evidenced by the retention of at least about 25% (e.g., at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more) activity under a pH that is between about 8.0 and about 11 (e.g., between about 8.0 and about 10, between about 8.5 and about 10.5, between about 8.5 and about 9.5, between about 9.5 and about 10.5, etc). In some embodiments, the improved activity/stability in the presence of a high concentration of bicarbonate is manifest or evidenced by the retention of at least about 25% (e.g., at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more) activity when the enzyme is incubated in the presence of about 0.1 M, 0.2 M, 0.5 M, 0.7 M, 0.9 M, 1.0 M, 1.2 M, 1.5 M, 2.0 M or a higher concentration of HCO_3^- for an extended period of time, for example, for at least 1 hour, 2 hours, 3 hours, 5 hours, 10 hours, 12 hours, 24 hours, several days, a week, several weeks, a month, or longer. In related embodiments, the carbonic anhydrase polypeptide has increased or higher thermostability at a higher ionic strength conditions. For example, the carbonic anhydrase polypeptide has a higher melting temperature at a higher ionic strength (e.g., 1 M bicarbonate) as compared to at a lower ionic strength (e.g., 0.8 M bicarbonate, 0.6 M bicarbonate, 0.4 M bicarbonate, 0.2 M bicarbonate, 0.1 M bicarbonate, etc). In certain embodiments, the recombinant carbonic anhydrase

comprises an amino acid sequence that is at least 55% identical to the amino acid sequence of SEQ ID NO: 136.

[0033] Carbon dioxide capture or sequestration processes that use carbonic anhydrase to convert CO₂ to bicarbonate typically involve the use of immobilized or soluble carbonic anhydrase in certain amine solvents or aqueous carbonate solutions. Thus, the Carbonic anhydrase polypeptides of the present disclosure in some aspects suitably have, in addition to good stability at high temperatures (e.g., at or above 40°C, 50°C, 60°C, 70°C, or 80°C), and/or high activity/stability or thermostability at high ionic strengths (e.g., at or about 0.5, 1.0, 1.5, 2.0, 2.5, or even 3.0 M bicarbonate), high stability when exposed to amine solvents (e.g., at or above concentrations of amine solvents typically used to immobilize or solublize carbonic anhydrase enzymes in industrial use to fix CO₂).

[0034] Furthermore, flue gases, which are the combustion exhaust gas produced at coal power plants typically can comprise, without limitation, oxygen, nitrogen, carbon dioxide, water vapor, along with small amounts of carbon monoxide, nitrogen oxides, sulfur oxides, and/or heavy metals, e.g., mercury. As such, the Carbonic anhydrase polypeptides of the present disclosure in certain aspects suitably have, in addition to good stability at high temperatures, and/or high activity/stability or thermostability at high ionic strengths, and/or high stability when exposed to amine solvents, also stability when exposed to oxides of sulfur, oxides of nitrogen, and/or to heavy metals, such as, e.g., mercury.

[0035] Moreover, natural gas compositions can vary depending on the sources from which the compositions have been extracted. For example, they may contain mostly methane and varying amounts of ethane, propane, butane, higher alkanes, nitrogen, carbon dioxide, and hydrogen sulfides. A number of previously known carbonic anhydrases can be destabilized when exposed to hydrogen sulfides. However, the Carbonic anhydrase polypeptides of the present disclosure, in some aspects, can suitably have, in addition to good stability at high temperatures, and/or high activity/stability or thermostability at high ionic strength, and/or high stability when exposed to amine solvents, and/or stability when exposed to oxides of sulfur, oxides of nitrogen, and/or to heavy metals, also good stability when exposed to hydrogen sulfides.

[0036] Also provided herein are an immobilized carbonic anhydrase enzyme or an immobilized variant thereof, and a method of preparing and/or using such an immobilized enzyme. In some embodiments, the carbonic anhydrase enzyme is immobilized using the various techniques provided herein. In certain embodiments, the carbonic anhydrase is a recombinant enzyme. In some embodiments, the stability and/or activity of carbonic anhydrase under a given condition (e.g., at a pH of between about 8.0 and about 10.0, at a temperature of between about 30°C and 85°C, or at an ionic strength conferred by at least about 0.1 M (e.g., at least about 0.2 M, at least about 0.5 M, at least about 1 M, at least about 1.5 M, at least about 2.0 M, or a higher concentration of) HCO₃⁻, is higher than that of a native (parent) carbonic anhydrase under the same condition.

[0037] These and other aspects of Carbonic anhydrase compositions and methods will be apparent from the following description.

DESCRIPTION OF THE DRAWINGS

[0038] Figure 1 provides a plasmid map of pHPLT02-BgiCA1.

5 [0039] Figures 2A-C provide temperature stability profiles of Bgi CA1 at pH values of (A) 8.5, (B) 9.5 and (C) 10.5, respectively.

[0040] Figure 3 provides a stability profile of Bgi CA1 in the presence of 1 M sodium bicarbonate (NaHCO₃).

10 [0041] Figure 4 provides an alignment of the amino acid sequence of the mature form of Bgi CA1 (SEQ ID NO: 3) with the sequences of other carbonic anhydrases (SEQ ID NOs: 16-25). Table 1G-1 lists the percent identity shared by the carbonic anhydrases of this alignment.

[0042] Figure 5 provides a phylogenetic tree for Bgi CA1 and its homologs.

[0043] Figure 6 provides a plasmid map of pJG183 (A4-celA-PviCA1).

15 [0044] Figures 7A-C provide temperature stability profiles of Pvi CA1 at pH values of (A) 8.5, (B) 9.5 and (C) 10.5, respectively.

[0045] Figure 8 provides a stability profile of Pvi CA1 in the presence of 1 M sodium bicarbonate (NaHCO₃).

20 [0046] Figure 9 provides an alignment of the amino acid sequence of the mature form of Pvi CA1 (SEQ ID NO: 28) with the sequences of other carbonic anhydrases (SEQ ID NOs: 16, 20, 21, 32-38). Table 2G-1 lists the percent identity shared by the carbonic anhydrases of this alignment

[0047] Figure 10 provides a phylogenetic tree for Pvi CA1 and its homologs.

[0048] Figure 11 provides a plasmid map of pTTT-AteCA1.

[0049] Figures 12A-C provide temperature stability profiles of Ate CA1 at pH values of (A) 8.5, (B) 9.5, and (C) 10.5, respectively.

25 [0050] Figure 13 provides a stability profile of Ate CA1 in the presence of 1 M sodium bicarbonate (NaHCO₃).

[0051] Figure 14 provides an alignment of the amino acid sequence of the mature form of Ate CA1 (SEQ ID NO: 43) with the sequences of other carbonic anhydrases (SEQ ID NOs: 45-48). Table 3G-1 lists the percent identity shared by the carbonic anhydrases of this alignment.

30 [0052] Figure 15 provides a phylogenetic tree for Ate CA1 and its homologs.

[0053] Figure 16 provides a plasmid map of pJG187 (A4-CelA-SprCA1).

- [0054] Figures 17A-C provide temperature stability profiles of Spr CA1 at pH values of (A) 8.5, (B) 9.5, and (C) 10.5, respectively.
- [0055] Figure 18 provides a stability profile of Spr CA1 in the presence of 1 M sodium bicarbonate (NaHCO₃).
- 5 [0056] Figure 19 provides an alignment of the amino acid sequence of the mature form of Spr CA1 (SEQ ID NO: 53) with the sequences of other carbonic anhydrases (SEQ ID NOS: 54-61). Table 4G-1 lists the percent identity shared by the carbonic anhydrases of this alignment.
- [0057] Figure 20 provides a phylogenetic tree for Spr CA1 and its homologs.
- [0058] Figure 21 provides a plasmid map of pHPLT02-BagCA1.
- 10 [0059] Figures 22A-C provide temperature stability profiles of Bag CA1 at pH values of (A) 8.5, (B) 9.5 and (C) 10.5, respectively.
- [0060] Figure 23 provides a stability profile of Bag CA1 in the presence of 1 M sodium bicarbonate (NaHCO₃).
- [0061] Figure 24 provides an alignment of the amino acid sequence of the mature form of Bag CA1
15 (SEQ ID NO: 64) with the sequences of other carbonic anhydrases (SEQ ID NOS: 16, 19, 21, 21, 22, 24, 25, 35, 74-76). Table 5G-1 lists the percent identity shared by the carbonic anhydrases of this alignment.
- [0062] Figure 25 provides a phylogenetic tree for Bag CA1 and its homologs.
- [0063] Figure 26 provides a plasmid map of pHPLT02-Vsp CA1.
- [0064] Figures 27A-C provide temperature stability profiles of Vsp CA1 at pH values of (A) 8.5,
20 (B) 9.5 and (C) 10.5, respectively.
- [0065] Figure 28 provides a stability profile of Vsp CA1 in the presence of 1 M sodium bicarbonate (NaHCO₃).
- [0066] Figure 29 provides a phylogenetic tree for Vsp CA1 and its homologs.
- [0067] Figure 30 provides a plasmid map of pHPLT02-VspE CA1.
- 25 [0068] Figure 31 provides a stability profile of VspE CA1 in the presence of 1 M sodium bicarbonate (NaHCO₃).
- [0069] Figure 32 provides a plasmid map of pHPLT02-VspE CA2.
- [0070] Figure 33 provides a stability profile of VspE CA2 in the presence of 1 M sodium bicarbonate (NaHCO₃).

DETAILED DESCRIPTION

I. Introduction

[0071] The present disclosure provides compositions and methods that relate to recombinant carbonic anhydrases from *Bacillus gibsonii*, *Promicromonospora vindobonensis*, *Aspergillus terreus*,
5 *Streptomyces pristinaespiralis*, *Bacillus agaradhaerens*, *Vibrio sp. AND4*, *Vibrio sp. Ex25*, polynucleotides encoding the carbonic anhydrases, and methods of make and/or use thereof.

[0072] In some embodiments, compositions and methods are described relating to a recombinant carbonic anhydrase from *Bacillus gibsonii* strain DSM 8722 (Bgi CA1). The compositions and methods are based, in part, on the observations that cloned and expressed Bgi CA1 is thermostable and/or retains
10 carbonic anhydrase activity under high-ionic strength conditions and/or in the presence of high concentrations of bicarbonate. These features of Bgi CA1 make it suitable for use in processes for extracting or sequestering carbon dioxide (CO₂) from, for example, combustion exhaust and flue gas streams.

[0073] The recombinant Bgi CA1 carbonic anhydrase has shown thermostability at pH values
15 ranging from about 8.5 to about 10.5. In particular, the recombinant Bgi CA1 retains about 90% of carbonic anhydrase activity when incubated for at least about 24 hours at 40°C at a pH of from about 8.5 to about 10.5, and about 40% of carbonic anhydrase activity when incubated for about 5 hours at 50°C, at a pH of from about 8.5 to about 9.5. Moreover, the recombinant Bgi CA1 retains over 70% of activity in a bicarbonate solution for at least about 3 hours. Additionally, the recombinant Bgi CA1 has a melting
20 temperature of at or above about 76°C under high ionic strength conditions in a bicarbonate solution. These characteristics make the Bgi CA1 described herein suitable for use in CO₂ extraction.

[0074] In some embodiments, compositions and methods are described relating to a recombinant carbonic anhydrase from *Promicromonospora vindobonensis* strain YIM65009 (Pvi CA1). The compositions and methods are based, in part, on the observations that cloned and expressed Pvi CA1 is
25 thermostable and/or retains carbonic anhydrase activity under high ionic strength conditions and/or in the presence of high concentrations of bicarbonate. These features of Pvi CA1 make it suitable for use in processes for extracting or sequestering carbon dioxide (CO₂) from, for example, combustion exhaust and flue gas streams.

[0075] The recombinant Pvi CA1 carbonic anhydrase has shown thermostability at pH values
30 ranging from about 8.5 to about 10.5. In particular, the recombinant Pvi CA1 retains greater than 60% of carbonic anhydrase activity when incubated for at least 24 hours at 40°C at a pH of about 8.5 to about 9.5. Moreover, the recombinant Pvi CA1 retains all of its carbon anhydrase activity in a bicarbonate solution for at least 3 hours at 50°C. Additionally, the recombinant Pvi CA1 has a melting temperature of at or above about 75°C under high ionic strength conditions.

[0076] In some embodiments, compositions and methods are described relating to a recombinant carbonic anhydrase cloned from *Aspergillus terreus* NIH2624 (Ate CA1). The compositions and methods are based, in part, on the observations that cloned and expressed Ate CA1 is thermostable and/or retains carbonic anhydrase activity under high-ionic strength conditions and/or in the presence of high concentrations of bicarbonate. These features of Ate CA1 make it suitable for use in processes for extracting or sequestering carbon dioxide (CO₂) from, for example, combustion exhaust and flue gas streams.

[0077] The recombinant Ate CA1 carbonic anhydrase has shown thermostability at pH values ranging from about 8.5 to about 10.5. In particular, the recombinant Ate CA1 retains about 60% or more (e.g., about 65% or more, about 70% or more, about 75% or more, about 80% or more, about 85% or more) of carbonic anhydrase activity when incubated for at least 24 hours at 40°C. The recombinant AteCA1 also may also retain about 50% or more (e.g., about 55% or more, about 60% or more) of carbonic anhydrase activity when incubated for about 1 hour at 50°C, at a pH of from about 8.5 to about 9.5. Moreover, the recombinant Ate CA1 retains about 70% or more (e.g., about 75% or more, about 80% or more, about 85% or more) of activity in the presence of bicarbonate for over 3 hours. Additionally, the recombinant Ate CA1 has a melting point of over about 74°C under high ionic strength conditions in a bicarbonate solution. Furthermore, the recombinant Ate CA1 can have a thermostability and/or melting temperature that is higher at a higher ionic strength condition (e.g., at 1 M bicarbonate) as compared to at a lower ionic strength condition (e.g., at 0.8, 0.6, 0.4, 0.2, or 0.1 M bicarbonate). These characteristics make the Ate CA1 described herein suitable for use in CO₂ extraction.

[0078] In some embodiments, compositions and methods are described relating to a recombinant carbonic anhydrase from *Streptomyces pristinaespiralis* strain ATCC 25486 (Spr CA1). The compositions and methods are based, in part, on the observations that cloned and expressed Spr CA1 is thermostable and/or retains carbonic anhydrase activity under high-ionic strength conditions and/or in the presence of high concentrations of bicarbonate. In some embodiments, the Spr CA1 polypeptides of the present disclosure have improved thermostability at higher ionic strength conditions. These features of Spr CA1 make it suitable for use in processes for extracting or sequestering carbon dioxide (CO₂) from, for example, combustion exhaust and flue gas streams.

[0079] The recombinant Spr CA1 carbonic anhydrase has shown thermostability at pH values ranging from about 8.5 to about 10.5. In particular, the recombinant Spr CA1 retains over 60% of carbonic anhydrase activity when incubated for at least 24 hours between 0°C and 40°C at a pH of between 8.5 and 9.5. Moreover, the recombinant Spr CA1 retains over 60% of activity in a bicarbonate solution at 50°C for at least about 2 hours. These characteristics make Spr CA1 suitable for use in carbon dioxide extraction.

[0080] In some embodiments, compositions and methods are described relating to a recombinant carbonic anhydrase from *Bacillus agaradhaerens* (Bag CA1). The compositions and methods are based,

in part, on the observations that cloned and expressed Bag CA1 is thermostable and/or retains carbonic anhydrase activity under high-ionic strength conditions and/or in the presence of high concentrations of bicarbonate. It is also observed that surprisingly the recombinant carbonic anhydrase has increased thermostability with increased ionic strength. These features of Bag CA1 make it suitable for use in processes for extracting or sequestering carbon dioxide (CO₂) from, for example, combustion exhaust and flue gas streams.

[0081] The recombinant Bag CA1 carbonic anhydrase has shown thermostability at pH values ranging from about 8.0 to about 11 (e.g., from about 8.5 to about 10.5, from about 8.5 to about 9.5, from about 9.5 to about 10.5, from about 8.5 to about 10.8, from about 8.6 to about 10.6, from about 8.4 to about 10.4, from about 8.2 to about 10.2, from about 8.0 to about 10, or from about 7.8 to about 9.8, etc). In particular, the recombinant Bag CA1 retains about 90% of carbonic anhydrase activity when incubated for at least about 3 hours, 5 hours, 10 hours, 24 hours or longer at about 25°C, and at a pH of from about 8.0 to about 11 (e.g., about 8.5 to about 10.5, about 8.5 to about 9.5, about 9.5 to about 10.5). The carbonic anhydrase activity decreased more markedly at higher pHs. However, at lower pHs (e.g., about 8 to about 9.5, about 8.5 to about 9.5, etc), Bag CA1 remains suitably stable over a long period of time at supra-physiological temperatures, at which enzymatic CO₂ extractions are conducted. Moreover, it has been observed that the recombinant Bag CA1 retained over 50% of activity in a bicarbonate solution for at least about 3 hours, at least about 5 hours, at least about 10 hours, at least about 24 hours, or longer. Additionally, the recombinant Bag CA1 has a melting temperature of at or above about 50°C (e.g., at least about 55°C, at least about 60°C, at least about 70°C, etc) under high ionic strength conditions, for example, in a bicarbonate solution. Furthermore, the Bag CA1 polypeptides of the present disclosure can suitably have improved thermostability, carbonic anhydrase activity, and/or higher melting temperatures at a higher ionic strength condition (for example, at 1 M bicarbonate concentration) as compared to these parameters, alone or in combination, at a lower ionic strength condition (for example, at 0.9 M bicarbonate concentration, at 0.8 M bicarbonate concentration, at 0.6 M bicarbonate concentration, at 0.5 M bicarbonate concentration, at 0.4 M bicarbonate concentration, at 0.3 M bicarbonate concentration, at 0.2 M bicarbonate concentration, or at 0.1 M bicarbonate concentration, etc. These characteristics make the Bag CA1 described herein suitable for use in CO₂ extraction.

[0082] In some embodiments, compositions and methods are described relating to a recombinant carbonic anhydrase from *Vibrio sp. AND4* (Vsp CA1). The compositions and methods are based, in part, on the observations that cloned and expressed Vsp CA1 is thermostable and/or retains carbonic anhydrase activity under high-ionic strength conditions and/or in the presence of high concentrations of bicarbonate. It has also been observed that the Vsp CA1 polypeptides of the present disclosure exhibit increased thermostability at higher ionic strength conditions, for example they suitably can have higher melting temperatures at higher ionic strength conditions, which is surprising. These features of Vsp CA1 make it suitable for use in processes for extracting or sequestering carbon dioxide (CO₂) from, for example, combustion exhaust and flue gas streams.

[0083] The recombinant Vsp CA1 carbonic anhydrase has shown thermostability at pH values ranging from about 8.0 to about 11 (e.g., about 8.5 to about 10.5, about 8.5 to about 9.5, about 9.5 to about 10.5, about 8.5 to about 10, etc). At the lower end of the basic pH, for example, at pH of about 8 to about 9.5 (e.g., about 8.5 to about 9.5, etc), the recombinant Vsp CA1 retains most of its carbonic anhydrase activity for an extended period of time, for example, for at least 1 hour, at least 2 hours, at least 3 hours, at least 5 hours, at least 10 hours, at least 24 hours, or even longer. For example, the recombinant Vsp CA1 suitably retains at least about 35% (e.g., at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70% or a higher percentage) of carbonic anhydrase activity when incubated for at least about 1 hours, 3 hours, 5 hours, 10 hours, or 24 hours or longer at 25°C at a pH of from about 8.5 to about 9.5. At the higher end of the basic pH, for example, at a pH of about 10.5 or higher, the recombinant enzymes loses its carbonic anhydrase activity much more rapidly. For example, the recombinant Vsp CA1 can suitably retain at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, or at least about 60% of its carbonic anhydrase activity when incubated for at least 1 hour, 2 hours or 3 hours at a pH of about 10.5. Moreover, the recombinant Vsp CA1 retains over 35% (e.g., over 35%, over 40%, over 45%, over 50%, over 55%, over 60%, over 65%, or even over 70%) of activity in a bicarbonate solution for at least about 1 hour, at least about 2 hours, or at least about 3 hours at 20°C. Additionally, the recombinant Vsp CA1 has a melting temperature of at or above about 65°C (e.g., at or above about 65°C, at or above about 66°C, at or above about 67°C, or at or above about 68°C, etc) under high ionic strength conditions, for example, in a bicarbonate solution. In related embodiments the recombinant Vsp CA1 can suitably have a higher or increased thermostability at higher ionic strength conditions. For example, the recombinant Vsp CA1 suitably has a higher melting temperature at a higher ionic strength (e.g., at 1 M bicarbonate) as compared to its melting temperature at a lower ionic strength (e.g., at 0.8 M bicarbonate, at 0.6 M bicarbonate, at 0.4 M bicarbonate, at 0.2 M bicarbonate, or at 0.1 M bicarbonate, etc). These characteristics make the Vsp CA1 described herein suitable for use in CO₂ extraction.

[0084] In some embodiments, compositions and methods are described relating to a recombinant carbonic anhydrase from *Vibrio sp. Ex25* (VspE CA1). The compositions and methods are based, in part, on the observations that cloned and expressed VspE CA1 is thermostable and/or retains carbonic anhydrase activity under high-ionic strength conditions and/or in the presence of high concentrations of bicarbonate. Moreover, the VspE CA1 polypeptides suitably have higher or improved thermostability at higher ionic strength conditions. These features of VspE CA1 make it suitable for use in processes for extracting or sequestering carbon dioxide (CO₂) from, for example, combustion exhaust and flue gas streams.

[0085] The recombinant VspE CA1 carbonic anhydrase has shown thermostability at pH values ranging from about 8 to about 11 (e.g., about 8.5 to about 10.5, about 8.5 to about 9.5, about 9.5 to about 10.5, about 8 to about 10, etc). In particular, the recombinant VspE CA1 retains at least about 55% (e.g.,

at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 97%, or 100%) of carbonic anhydrase activity when incubated for at least about 30 minutes, about 1 hour, about 2 hours, about 5 hours, about 10 hours, about 15 hours, or about 24 hours, or longer at about 25°C, or about 50°C, at a pH of from about 8 to about 11 (e.g., about 8.5 to about 10.5, about 8.5 to about 9.5, about 9.5 to about 10.5, about 8 to about 10, etc). Moreover, the recombinant VspE CA1 retains over 30% of activity in a bicarbonate solution for at least about 30 minutes, about 1 hour, about 2 hours, or about 3 hours or longer at about 20°C, about 25°C, or about 40°C. Additionally, the recombinant VspE CA1 has a melting temperature of at or above about 45°C (e.g., at or above about 45°C, about 46°C, about 47°C, about 48°C, about 49°C, or about 50°C, or higher, under high ionic strength conditions in a bicarbonate solution. In some embodiments, the recombinant VspE CA1 has an increased or higher thermostability at higher ionic strength conditions. For example, the recombinant VspE CA1 suitably has a higher melting temperature at a higher ionic strength (e.g., 1 M bicarbonate) as compared to at a lower ionic strength (e.g., 0.8 M bicarbonate, 0.6 M bicarbonate, 0.4 M bicarbonate, 0.2 M bicarbonate, or 0.1 M bicarbonate, etc). These characteristics make the VspE CA1 described herein suitable for use in CO₂ extraction.

[0086] In some embodiments, compositions and methods are described relating to a recombinant carbonic anhydrase II from *Vibrio sp. Ex25* (VspE CA2). The compositions and methods are based, in part, on the observations that cloned and expressed VspE CA2 is thermostable and/or retains carbonic anhydrase activity under high-ionic strength conditions and/or in the presence of high concentrations of bicarbonate. Moreover, the VspE CA2 also has higher thermostability at higher ionic strength conditions. These features of VspE CA2 make it suitable for use in processes for extracting or sequestering carbon dioxide (CO₂) from, for example, combustion exhaust and flue gas streams.

[0087] The recombinant VspE CA2 carbonic anhydrase has shown thermostability at pH values ranging from about 8 to about 11 (e.g., about 8.5 to about 10.5, about 8 to about 10, about 8.4 to about 9.5, about 9.5 to about 10.5, etc.). In particular, the recombinant VspE CA2 retains at least about 25% (e.g., at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, or a higher percentage) of carbonic anhydrase activity when incubated for at least 30 minutes, at least 1 hour, at least 2 hours, at least 3 hours, at least 5 hours, at least 10 hours, at least 24 hours at 25°C at a pH of from about 8 to about 11 (e.g., about 8.5 to about 10.5, about 8 to about 10, about 8.4 to about 9.5, about 9.5 to about 10.5, etc). Moreover, the recombinant VspE CA2 retains over 40% (e.g., over 40%, over 45%, over 50%, over 55%, over 60%, over 65%, over 70%, over 75%, over 80%, or a higher percentage) of activity in a bicarbonate solution for at least about 30 minutes, about 1 hour, about 2 hours, or about 3 hours at 20°C. Additionally, the recombinant VspE CA2 has a melting temperature of at or above about 45°C (e.g., at or above 47°C, at or about 50°C, at or above 65°C, at or above 70°C, at or above 75°C, or higher temperature) under high ionic strength conditions in a bicarbonate solution. Importantly the recombinant VspE CA2 has an increased or higher thermostability at a higher ionic strength condition.

For example, the recombinant VspE CA2 can suitably have a higher melting temperature at a higher ionic strength (e.g., 1 M bicarbonate) as compared to at a lower ionic strength (e.g., 0.8 M bicarbonate, 0.6 M bicarbonate, 0.4 M bicarbonate, 0.2 M bicarbonate, 0.1 M bicarbonate, etc.). These characteristics make the VspE CA2 described herein suitable for use in CO₂ extraction.

5 II. Definitions

[0088] Prior to describing the present compositions and methods in detail, the following terms are defined for clarity. Terms and abbreviations not defined should be accorded their ordinary meaning as used in the art:

10 [0089] As used herein, “carbonic anhydrase” or “CA” describes a polypeptide having EC 4.2.1.1 activity, which is capable of catalyzing the inter-conversion between carbon dioxide and bicarbonate [i.e., $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+$].

[0090] The term “CO₂-containing medium,” describes any material that may contain at least 0.001% CO₂, carbonic acid, bicarbonate, carbonate, or mixtures thereof, or at least 0.01%, at least 0.1%, at least 1%, at least 5%, at least 10%, at least 20%, at least 50%, or more CO₂, carbonic acid, bicarbonate,
 15 carbonate, or mixtures thereof. For brevity sake, references made to a CO₂-containing medium encompass a medium containing carbonic acid, bicarbonate, carbonate or mixtures thereof, in addition to or in the absence of CO₂. Carbon dioxide-containing media may be gaseous phases, liquids, or multiphase mixtures, but may also be solids. A CO₂-containing gaseous phase is, for example, raw natural gas obtainable from oil wells, gas wells, and condensate wells, syngas generated by the
 20 gasification of a carbon containing fuel (e.g., methane) to a gaseous product comprising CO and/or H₂, or emission streams from combustion processes, e.g., from carbon-based electricity generation power plants, from flue gas stacks from such plants, industrial furnaces, stoves, ovens, or fireplaces, or from airplane or car exhausts. A CO₂-containing gaseous phase may alternatively be from respiratory processes in mammals, living plants and other CO₂ emitting species, in particular that from greenhouses.
 25 A CO₂-containing gas phase may also be an off-gas, from aerobic or anaerobic fermentation, such as brewing, fermentation to produce useful products such as ethanol, or the production of biogas. Such fermentation processes can occur at elevated temperatures if they are facilitated by thermophilic microorganisms, which are, for example, seen in the production of biogas. A CO₂-containing gaseous phase may alternatively be a gaseous phase enriched in CO₂ for the purpose of use or storage. The above
 30 described gaseous phases may also occur as multiphase mixtures, where the gas co-exists with a certain degree of fluids (e.g., water or other solvents) and/or solid materials (e.g., ash or other particles). Carbon dioxide-containing liquids are any solutions or fluids, in particular aqueous liquids, containing measurable amounts of CO₂. Carbon dioxide-containing liquids may be obtained by passing a CO₂-containing gas or solid (e.g., dry ice or soluble carbonate containing salt) into the liquids. A carbon
 35 dioxide-containing fluid may also be a compressed CO₂ liquid (that contains contaminants, such as a dry-cleaning fluid), or a supercritical CO₂, or a CO₂ solvent liquid, such as an ionic liquid.

[0091] The term “carbon dioxide extraction” or “CO₂ extraction” describes a reduction or otherwise separation of CO₂ from a CO₂-containing medium. Such an extraction may be performed from one medium to another, *e.g.*, gas to liquid, liquid to gas, gas to liquid to gas, liquid to liquid, or liquid to solid, but the extraction may also refer to the conversion of CO₂ to bicarbonate or carbonate, or the conversion of bicarbonate or carbonate to CO₂, within the same medium. The terms “CO₂ capture” and “CO₂ sequestration” are also used to indicate extraction of CO₂ from one medium to another, separating CO₂ from a CO₂-containing medium, conversion of CO₂ to bicarbonate or carbonate, or conversion of bicarbonate or carbonate to CO₂.

[0092] The term “syngas” or “synthesis gas,” describes a gas mixture that contains varying amounts of carbon monoxide (CO) and hydrogen (H₂) generated by the gasification of a carbon-containing fuel (*e.g.*, methane or natural gas) to a gaseous product with a heating value. Carbon dioxide is produced in the syngas reaction and is removed to increase the heating value.

[0093] As used herein, “ionic strength conditions,” with reference to a protein, refers to incubation of the protein in a solution containing an ionic salt, such as, for example, sodium chloride, potassium chloride, calcium chloride, sodium bicarbonate, potassium bicarbonate, ammonium bicarbonate, sodium carbonate, potassium carbonate, ammonium carbonate, etc. When the salt is present at a high concentration (*e.g.*, 0.1 M, 0.5 M, 1 M, 1.5 M, 2 M, etc.), then the protein is said to be incubated under high ionic strength conditions.

[0094] As used herein, “catalytic activity” or “activity” describes quantitatively the conversion of a given substrate under defined reaction conditions. The term “residual activity” is defined as the ratio of the catalytic activity of the enzyme under a certain set of conditions to the catalytic activity under a different set of conditions. The term “specific activity” describes quantitatively the catalytic activity per amount of enzyme under defined reaction conditions.

[0095] As used herein, “pH-stability,” with reference to a protein, refers to the ability of a protein to withstand a limited exposure to pH values significantly deviating from the pH where its stability is optimal (*e.g.*, more than one pH unit above or below the pH optimum, without significantly losing its activity measured under conditions where its activity is measurable).

[0096] The term “thermal stability” or “thermostable,” when used in reference to a carbonic anhydrase, refers to a carbonic anhydrase that retain a specified amount of enzymatic activity after exposure to an identified altered temperature over a given period of time under conditions prevailing during the carbon dioxide hydration, bicarbonate dehydration, or other process disclosed herein. Altered temperatures include increased or decreased temperatures from a temperature under which the native enzyme typically operates. In some embodiments, the identified altered temperature is, for example, a temperature within the range of about 20°C to about 80°C, for example, about 30°C to about 70°C, or about 40°C to about 65°C. In some embodiments, the carbonic anhydrase retains at least about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 75%, about 80%, about 85%, about

90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% of a carbonic anhydrase activity after exposure to an altered temperature over a given time period, for example, for at least about 15 minutes, about 30 minutes, about 60 minutes, about 120 minutes, about 180 minutes, about 240 minutes, about 300 minutes, a month, several months, a year, several years etc.

[0097] As used herein, the term “purified” or “isolated” refers to the physical separation of a subject molecule, such as Bgi CA1, from its native source (*e.g.*, *Bacillus gibsonii*) or other molecules, such as proteins, nucleic acids, lipids, media components, and the like. Once purified or isolated, a subject molecule may represent at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, or more, of the total amount of material in a sample (wt/wt).

[0098] As used herein, a “polypeptide” refers to a molecule comprising a plurality of amino acids linked through peptide bonds. The terms “polypeptide,” “peptide,” and “protein” are used interchangeably. Proteins may optionally be modified (*e.g.*, glycosylated, phosphorylated, acylated, farnesylated, prenylated, sulfonated, and the like) to add functionality. Where such amino acid sequences exhibit activity, they may be referred to as an “enzyme.” The conventional one-letter or three-letter codes for amino acid residues are used, with amino acid sequences being presented in the standard amino-to-carboxy terminal orientation (*i.e.*, N→C).

[0099] As used herein, an “immobilized polypeptide” refers to a polypeptide that is physically confined on an immobilization material while retaining its function or catalytic activity. Examples of immobilization materials include, without limitation, a polymer, a membrane including liquid membranes, a matrix, a wafer, a solid support, or a micro-particle. In some embodiments, an “immobilized polypeptide” describes a polypeptide that is physically confined, which has increased stability and/or catalytic activity.

[00100] The term “polynucleotide” encompasses DNA, RNA, heteroduplexes, and synthetic molecules capable of encoding a polypeptide. Nucleic acids may be single-stranded or double-stranded, and may have chemical modifications. The terms “nucleic acid” and “polynucleotide” are used interchangeably. Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and the present compositions and methods encompass nucleotide sequences which encode a particular amino acid sequence. Unless otherwise indicated, nucleic acid sequences are presented in a 5'-to-3' orientation.

[00101] As used herein, the term “wild-type” or “native” refers to polypeptides or polynucleotides that are found in nature.

[00102] The terms “wild-type,” “parental,” or “reference,” with respect to a polypeptide, refer to a naturally-occurring polypeptide that does not include a man-made substitution, insertion, or deletion at one or more amino acid positions. Similarly, the term “wild-type,” “parental,” or “reference,” with

respect to a polynucleotide, refers to a naturally-occurring polynucleotide that does not include a man-made nucleoside change. However, a polynucleotide encoding a wild-type, parental, or reference polypeptide is not limited to a naturally-occurring polynucleotide, but rather encompasses any polynucleotide encoding the wild-type, parental, or reference polypeptide.

5 **[00103]** As used herein, a “variant polypeptide” refers to a polypeptide that is derived from a parent (or reference) polypeptide by the substitution, addition, or deletion, of one or more amino acids, typically by recombinant DNA techniques. Variant polypeptides may differ from a parent polypeptide by a small number of amino acid residues. They may be defined by their level of primary amino acid sequence homology/identity with a parent polypeptide. Suitably, variant polypeptides have at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or even at least 99% amino acid sequence identity to a parent polypeptide.

10 **[00104]** Sequence identity may be determined using known programs such as BLAST, ALIGN, and CLUSTAL using standard parameters. (*See, e.g., Altschul et al. J. Mol. Biol.* 215:403-410, 1990; Henikoff *et al. Proc. Natl. Acad. Sci. USA* 89:10915, 1989; Karin *et al. Proc. Natl. Acad. Sci. USA* 90:5873, 1993; and Higgins *et al. Gene* 73:237-244, 1988). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. Databases may also be searched using FASTA (Pearson *et al. Proc. Natl. Acad. Sci. USA* 85:2444-2448, 1988). One indication that two polypeptides are substantially identical is that the first polypeptide is immunologically cross-reactive with the second polypeptide. Typically, polypeptides that differ by conservative amino acid substitutions are immunologically cross-reactive. Thus, a polypeptide is substantially identical to a second polypeptide, for example, where the two peptides differ only by a conservative substitution.

25 **[00105]** As used herein, a “variant polynucleotide” encodes a variant polypeptide, has a specified degree of homology/identity with a parent polynucleotide, or hybridized under stringent conditions to a parent polynucleotide or the complement thereof. Suitably, a variant polynucleotide has at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or even at least 99% nucleotide sequence identity to a parent polynucleotide or to a complement of the parent polynucleotide. Methods for determining percent identity are known in the art and described above.

30 **[00106]** The term “derived from” encompasses the terms “originated from,” “obtained from,” “obtainable from,” “isolated from,” and “created from,” and generally indicates that one specified material find its origin in another specified material or has features that can be described with reference to the another specified material.

35 **[00107]** As used herein, the term “hybridization” refers to the process by which a strand of nucleic acid joins with a complementary strand through base pairing, as known in the art.

[00108] As used herein, the phrase “hybridization conditions” refers to the conditions under which hybridization reactions are conducted. These conditions are typically classified by degree of “stringency” of the conditions under which hybridization is measured. The degree of stringency can be based, for example, on the melting temperature (T_m) of the nucleic acid binding complex or probe. For example, “maximum stringency” typically occurs at about $T_m - 5^\circ\text{C}$ (5°C below the T_m of the probe); “high stringency” at about $5-10^\circ\text{C}$ below the T_m ; “intermediate stringency” at about $10-20^\circ\text{C}$ below the T_m of the probe; and “low stringency” at about $20-25^\circ\text{C}$ below the T_m . Alternatively, or in addition, hybridization conditions can be based upon the salt or ionic strength conditions of hybridization, and/or upon one or more stringency washes, *e.g.*: 6X SSC = very low stringency; 3X SSC = low to medium stringency; 1X SSC = medium stringency; and 0.5X SSC = high stringency. Functionally, maximum stringency conditions may be used to identify nucleic acid sequences having strict identity or near-strict identity with the hybridization probe; while high stringency conditions are used to identify nucleic acid sequences having about 80% or more sequence identity with the probe. For applications requiring high selectivity, it is typically desirable to use relatively stringent conditions to form the hybrids (*e.g.*, relatively low salt and/or high temperature conditions are used). As used herein, stringent conditions are defined as 50°C and 0.2X SSC (1X SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0).

[00109] The phrase “substantially similar” or “substantially identical,” in the context of at least two nucleic acids or polypeptides, means that a polynucleotide or polypeptide comprises a sequence that has at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or even at least about 99% identical to a parent or reference sequence, or does not include amino acid substitutions, insertions, deletions, or modifications made only to circumvent the present description without adding functionality.

[00110] As used herein, an “expression vector” refers to a DNA construct containing a DNA sequence that encodes a specified polypeptide and is operably linked to a suitable control sequence capable of effecting the expression of the polypeptides in a suitable host. Such control sequences may include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites and/or sequences that control termination of transcription and translation. The vector may be a plasmid, a phage particle, or a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the host genome.

[00111] The term “recombinant,” refers to genetic material (*i.e.*, nucleic acids, the polypeptides they encode, and vectors and cells comprising such polynucleotides) that has been modified to alter its sequence or expression characteristics, such as by mutating the coding sequence to produce an altered polypeptide, fusing the coding sequence to that of another gene, placing a gene under the control of a different promoter, expressing a gene in a heterologous organism, expressing a gene at a decreased or elevated levels, expressing a gene conditionally or constitutively in a manner different from its natural

expression profile, and the like. Generally recombinant nucleic acids, polypeptides, and cells based thereon, have been manipulated by man such that they are not identical to related nucleic acids, polypeptides, and cells found in nature.

5 [00112] A “signal sequence” refers to a sequence of amino acids bound to the N-terminal portion of a polypeptide, and which facilitates the secretion of the mature form of the protein from the cell. The mature form of the extracellular protein lacks the signal sequence which is cleaved off during the secretion process.

[00113] The term “selective marker” or “selectable marker,” refers to a gene capable of expression in a host cell that allows for ease of selection of those hosts containing an introduced nucleic acid or vector.
10 Examples of selectable markers include but are not limited to antimicrobial substances (*e.g.*, hygromycin, bleomycin, or chloramphenicol) and/or genes that confer a metabolic advantage, such as a nutritional advantage, on the host cell.

[00114] The term “regulatory element,” refers to a genetic element that controls some aspect of the expression of nucleic acid sequences. For example, a promoter is a regulatory element which facilitates
15 the initiation of transcription of an operably linked coding region. Additional regulatory elements include splicing signals, polyadenylation signals and termination signals.

[00115] As used herein, “host cells” are generally cells of prokaryotic or eukaryotic hosts that are transformed or transfected with vectors constructed using recombinant DNA techniques known in the art. Transformed host cells are capable of either replicating vectors encoding the protein variants or
20 expressing the desired protein variant. In the case of vectors, which encode the pre- or pro-form of the protein variant, such variants, when expressed, are typically secreted from the host cell into the host cell medium.

[00116] The term “introduced,” in the context of inserting a nucleic acid sequence into a cell, means transformation, transduction, or transfection. Means of transformation include protoplast transformation,
25 calcium chloride precipitation, electroporation, naked DNA, and the like as known in the art. (*See*, Chang and Cohen *Mol. Gen. Genet.* 168:111-115, 1979; Smith *et al. Appl. Env. Microbiol.* 51:634, 1986; and the review article by Ferrari *et al.*, in Harwood, *Bacillus*, Plenum Publishing Corporation, pp. 57-72, 1989).

[00117] Other technical and scientific terms have the same meaning as commonly understood by one
30 of ordinary skill in the art to which this disclosure pertains (*See, e.g.*, Singleton and Sainsbury, *Dictionary of Microbiology and Molecular Biology*, 2d Ed., John Wiley and Sons, NY 1994; and Hale and Marham, *The Harper Collins Dictionary of Biology*, Harper Perennial, NY 1991).

[00118] The singular terms “a,” “an,” and “the” include the plural reference unless the context clearly indicates otherwise.

[00119] As used herein in connection with a numerical value, the term “about” refers to a range of -10% to +10% of the numerical value, unless the term is otherwise specifically defined in context. For instance, the phrase a “pH value of about 6” refers to pH values of from 5.4 to 6.6, unless the pH value is specifically defined otherwise.

- 5 [00120] Headings are provided for convenience and should not be construed as limitations. The description included under one heading may apply to the specification as a whole.

III. Carbonic Anhydrases Polypeptides, Polynucleotides, Vectors, and Host Cells

A. Carbonic Anhydrases Polypeptides

10 [00121] In one aspect, the present compositions and methods provide a recombinant Bgi CA1 carbonic anhydrase polypeptide, fragments thereof, or variants thereof having carbonic anhydrase activity. An example of a recombinant Bgi CA1 polypeptide was isolated from *Bacillus gibsonii* strain DSM 8722. The mature Bgi CA1 polypeptide has the amino acid sequence set forth as SEQ ID NO: 3. Similar, substantially similar Bgi CA1 polypeptides may occur in nature, *e.g.*, in other strains or isolates of *B. gibsonii*. These and other recombinant Bgi CA1 polypeptides are encompassed by the present
15 compositions and methods.

[00122] In one aspect, the present compositions and methods provide a recombinant Pvi CA1 carbonic anhydrase polypeptide, fragments thereof, or variants thereof having carbonic anhydrase activity. An example of a recombinant Pvi CA1 polypeptide was isolated from *Promicromonospora vindobonensis* strain YIM65009. The mature Pvi CA1 polypeptide has the amino acid sequence set forth
20 as SEQ ID NO: 28. Similar, substantially similar Pvi CA1 polypeptides may occur in nature, *e.g.*, in other strains or isolates of *P. vindobonensis*. These and other recombinant Pvi CA1 polypeptides are encompassed by the present compositions and methods.

[00123] In one aspect, the present compositions and methods provide a recombinant Ate CA1 carbonic anhydrase polypeptide, fragments thereof, or variants thereof having carbonic anhydrase
25 activity. An example of a recombinant Ate CA1 polypeptide was isolated from *Aspergillus terreus* strain NIH2624. The mature Ate CA1 polypeptide has the amino acid sequence set forth as SEQ ID NO: 43. Similar, substantially similar Ate CA1 polypeptides may occur in nature, *e.g.*, in other strains or isolates of *A. terreus*. These and other recombinant Ate CA1 polypeptides are encompassed by the present compositions and methods.

30 [00124] In one aspect, the present compositions and methods provide a recombinant Spr CA1 carbonic anhydrase polypeptide, fragments thereof, or variants thereof having carbonic anhydrase activity. An example of a recombinant Spr CA1 polypeptide was isolated from *Streptomyces pristinaespiralis* strain ATCC 25486. The mature Spr CA1 polypeptide has the amino acid sequence set forth as SEQ ID NO: 53. Similar, substantially similar Spr CA1 polypeptides may occur in nature, *e.g.*,

in other strains or isolates of *S. pristinaespiralis*. These and other recombinant Spr CA1 polypeptides are encompassed by the present compositions and methods.

[00125] In one aspect, the present compositions and methods provide a recombinant Bag CA1 carbonic anhydrase polypeptide, fragments thereof, or variants thereof having carbonic anhydrase activity. An example of a recombinant Bag CA1 polypeptide was isolated from *Bacillus agaradhaerens*. The mature Bag CA1 polypeptide has the amino acid sequence set forth as SEQ ID NO: 64. Similar, substantially similar Bag CA1 polypeptides may occur in nature, *e.g.*, in other strains or isolates of *B. agaradhaerens*. These and other recombinant Bag CA1 polypeptides are encompassed by the present compositions and methods.

[00126] In one aspect, the present compositions and methods provide a recombinant Vsp CA1 carbonic anhydrase polypeptide, fragments thereof, or variants thereof having carbonic anhydrase activity. An example of a recombinant Vsp CA1 polypeptide was isolated from *Vibrio sp. AND4*. The mature Vsp CA1 polypeptide has the amino acid sequence set forth as SEQ ID NO: 80. Similar, substantially similar Vsp CA1 polypeptides may occur in nature, *e.g.*, in other strains or isolates of *Vibrio sp. AND4*. These and other recombinant Vsp CA1 polypeptides are encompassed by the present compositions and methods.

[00127] In one aspect, the present compositions and methods provide a recombinant VspE CA1 carbonic anhydrase polypeptide, fragments thereof, or variants thereof having carbonic anhydrase activity. An example of a recombinant VspE CA1 polypeptide was isolated from *Vibrio sp. Ex25*. The mature VspE CA1 polypeptide has the amino acid sequence set forth as SEQ ID NO: 130. Similar, substantially similar VspE CA1 polypeptides may occur in nature, *e.g.*, in other strains or isolates of *Vibrio sp. Ex25*. These and other recombinant VspE CA1 polypeptides are encompassed by the present compositions and methods.

[00128] In one aspect, the present compositions and methods provide a recombinant VspE CA2 carbonic anhydrase polypeptide, fragments thereof, or variants thereof having carbonic anhydrase activity. An example of a recombinant VspE CA2 polypeptide was isolated from *Vibrio sp. Ex25*. The mature VspE CA2 polypeptide has the amino acid sequence set forth as SEQ ID NO: 136. Similar, substantially similar VspE CA2 polypeptides may occur in nature, *e.g.*, in other strains or isolates of *Vibrio sp. Ex25*. These and other recombinant VspE CA2 polypeptides are encompassed by the present compositions and methods.

[00129] In some embodiments, the recombinant polypeptide is a variant polypeptide having a specified degree of amino acid sequence identity to the exemplified parent polypeptide, *e.g.*, at least 50%, at least 51%, at least 52%, at least 53%, at least 54%, at least 55%, at least 56%, at least 57%, at least 58%, at least 59%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or even at least 99% sequence identity

to the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 28, SEQ ID NO: 43, SEQ ID NO: 53, SEQ ID NO: 64, SEQ ID NO: 80, SEQ ID NO: 130, or SEQ ID NO: 136. Sequence identity can be determined by amino acid sequence alignment, *e.g.*, using a program such as BLAST, ALIGN, or CLUSTAL, as described herein.

- 5 **[00130]** In certain embodiments, the recombinant polypeptides are produced recombinantly, while in others the polypeptides are produced synthetically, or are purified from a native source (*e.g.*, *B. gibsonii*, *Promicromonospora vindobonensis*, *Aspergillus terreus*, *Streptomyces pristinaespiralis*, *Bacillus agaradhaerens*, *Vibrio sp. AND4*, or *Vibrio sp. Ex25*).

- 10 **[00131]** In certain embodiments, the recombinant polypeptides include substitutions that do not substantially affect the structure and/or function of the polypeptide. Examples of these substitutions are conservative mutations, as summarized in Table I.

Table I. Amino Acid Substitutions

Original Residue	Code	Acceptable Substitutions
Alanine	A	D-Ala, Gly, beta-Ala, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, beta-Ala, Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Leu, D-Leu, Met, D-Met
Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, cis-3,4, or 5-phenylproline
Proline	P	D-Pro, L-I-thioazolidine-4- carboxylic acid, D-or L-1-oxazolidine-4- carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val

Original Residue	Code	Acceptable Substitutions
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

[00132] Substitutions involving naturally occurring amino acids are generally made by mutating a nucleic acid encoding a recombinant carbonic anhydrase polypeptide, and then expressing the variant polypeptide in an organism. Substitutions involving non-naturally occurring amino acids or chemical modifications to amino acids are generally made by chemically modifying a carbonic anhydrase polypeptide after it has been synthesized by an organism.

[00133] In some embodiments, variant recombinant polypeptides are substantially identical to SEQ ID NO: 3, SEQ ID NO: 28, SEQ ID NO: 43, SEQ ID NO: 53, SEQ ID NO: 64, SEQ ID NO: 80, SEQ ID NO: 130, or SEQ ID NO: 136, meaning that they do not include amino acid substitutions, insertions, or deletions that do not significantly affect the structure, function, or expression of the polypeptide. Such variant recombinant carbonic anhydrase polypeptides will include those designed to circumvent the present description. In some embodiments, variants recombinant polypeptides, compositions and methods comprising these variants are not substantially identical SEQ ID NO: 3, SEQ ID NO: 28, SEQ ID NO: 43, SEQ ID NO: 53, SEQ ID NO: 64, SEQ ID NO: 80, SEQ ID NO: 130, or SEQ ID NO: 136, but rather include amino acid substitutions, insertions, or deletions that affect, in certain circumstances, substantially, the structure, function, or expression of the polypeptide herein such that improved characteristics, including, e.g., improved specific activity, improved thermostability, improved pH stability, and/or improved activity under high ionic strength conditions, as compared to that of a polypeptide of SEQ ID NO: 3, SEQ ID NO: 28, SEQ ID NO: 43, SEQ ID NO: 53, SEQ ID NO: 64, SEQ ID NO: 80, SEQ ID NO: 130, or SEQ ID NO: 136, can be achieved.

[00134] In some embodiments, the recombinant polypeptide (including a variant thereof) has carbonic anhydrase activity. Carbonic anhydrase activity can be determined and measured using the assays described herein, or by other assays known in the art. In some embodiments, the recombinant carbonic anhydrase polypeptide has activity in the presence of a composition used to extract or sequester carbon dioxide.

[00135] Recombinant polypeptides include fragments of the “full-length” polypeptides that retain carbonic anhydrase activity. Such fragments suitably retain the active site of the full-length polypeptides but may have deletions of non-critical amino acid residues. The activity of fragments can be readily determined using the assays described herein, or by other assays known in the art. In some embodiments, the fragments of the polypeptides retain carbonic anhydrase activity in the presence of a composition used to extract or sequester carbon dioxide.

[00136] In some embodiments, the amino acid sequences and derivatives are produced as an N- and/or C-terminal fusion protein, for example, to aid in extraction, detection and/or purification and/or to add functional properties to the polypeptides. Examples of fusion protein partners include, but are not limited to, glutathione-S-transferase (GST), 6XHis, GAL4 (DNA binding and/or transcriptional activation domains), FLAG-, MYC-tags or other tags known to those skilled in the art. In some embodiments, a proteolytic cleavage site is provided between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences. Suitably, the fusion protein does not hinder the activity of the recombinant polypeptide. In some embodiments, the recombinant polypeptide is fused to a functional domain including a leader peptide, propeptide, binding domain and/or catalytic domain. Fusion proteins are optionally linked to the recombinant polypeptide through a linker sequence that joins the polypeptide and the fusion domain without significantly affecting the properties of either component. The linker optionally contributes functionally to the intended application.

[00137] In some embodiments, the recombinant polypeptide is expressed in a heterologous organism, *i.e.*, an organism other than the native source. Examples of suitable heterologous organisms are Gram(+) bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Geobacillus* (formerly *Bacillus*) *stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, *Streptomyces lividans*, or *Streptomyces murinus*; Gram(-) bacteria such as *Escherichia coli*; yeast such as *Saccharomyces* spp. or *Schizosaccharomyces* spp., *e.g.* *Saccharomyces cerevisiae*; and filamentous fungi such as *Aspergillus* spp., *e.g.*, *Aspergillus oryzae* or *Aspergillus niger*, and *Trichoderma reesei*. Methods of transforming nucleic acids into these organisms are known in the art. For example, a suitable procedure for transforming *Aspergillus* host cells is described in EP 238 023.

[00138] In some embodiments, the recombinant polypeptide is fused to a signal peptide, for example, to facilitate extracellular secretion of the recombinant polypeptide. For example, in certain embodiments, the signal peptide is encoded by a sequence selected from SEQ ID NOs: 10, 11, 12, 13, 14, 15, 69, 70, 71, 72, 73, 81, 82, 83, 131 and 132. In particular embodiments, the recombinant polypeptide is expressed in a heterologous organism as a secreted polypeptide. The compositions and methods herein thus encompass methods for expressing a carbonic anhydrase polypeptide as a secreted polypeptide in a heterologous organism.

B. Carbonic Anhydrase Polynucleotides

[00139] Another aspect of the compositions and methods described herein is a polynucleotide that encodes a recombinant carbonic anhydrase polypeptide (including variants and fragments thereof) having carbonic anhydrase activity. In some embodiments the polynucleotide is provided in the context of an expression vector for directing the expression of a carbonic anhydrase polypeptide in a heterologous organism, such as the ones identified herein. The polynucleotide that encodes a recombinant carbonic

anhydrase polypeptide may be operably-linked to regulatory elements (*e.g.*, a promoter, terminator, enhancer, and the like) to assist in expressing the encoded polypeptides.

[00140] Examples of polynucleotide sequences encoding recombinant carbonic anhydrase polypeptides are the nucleotide sequence of SEQ ID NO: 1, SEQ ID NO: 26, SEQ ID NO: 41 SEQ ID NO: 51, SEQ ID NO: 62, SEQ ID NO: 77, SEQ ID NO: 127, or SEQ ID NO: 133. Similar, including substantially identical, polynucleotides encoding recombinant carbonic anhydrase polypeptides and variants may occur in nature, *e.g.*, in different strains or other isolates of the native source. In view of the degeneracy of the genetic code, it will be appreciated that polynucleotides having different nucleotide sequences may encode the same carbonic anhydrase polypeptides, variants, or fragments.

[00141] In some embodiments, polynucleotides encoding recombinant polypeptides have a specified degree of amino acid sequence identity to the exemplified polynucleotide encoding a parent polypeptide, *e.g.*, at least 50%, at least 51%, at least 52%, at least 53%, at least 54%, at least 55%, at least 56%, at least 57%, at least 58%, at least 59%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or even at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 28, SEQ ID NO: 43, SEQ ID NO: 53, SEQ ID NO: 64, SEQ ID NO: 80, SEQ ID NO: 130, or SEQ ID NO: 136. Homology can be determined by amino acid sequence alignment, *e.g.*, using a program such as BLAST, ALIGN, or CLUSTAL, as described herein.

[00142] In some embodiments, the polynucleotide that encodes a recombinant polypeptide is fused in frame behind (*i.e.*, downstream of) a coding sequence for a signal peptide for directing the extracellular secretion of a recombinant polypeptide. Heterologous signal sequences include, for example, those from bacterial cellulase genes. Expression vectors may be provided in a heterologous host cell suitable for expressing a recombinant polypeptide, or suitable for propagating the expression vector prior to introducing it into a suitable host cell.

[00143] In some embodiments, polynucleotides encoding recombinant polypeptides hybridize to the polynucleotide of SEQ ID NO: 1, SEQ ID NO: 26, SEQ ID NO: 41 SEQ ID NO: 51, SEQ ID NO: 62, SEQ ID NO: 77, SEQ ID NO: 127, or SEQ ID NO: 133 (or to the complement thereof) under specified hybridization conditions. Examples of conditions are intermediate stringency, high stringency and extremely high stringency conditions, which are described, herein.

[00144] Carbonic Anhydrase polynucleotides may be naturally occurring or synthetic (*i.e.*, man-made), and may be codon-optimized for expression in a different host, mutated to introduce cloning sites, or otherwise altered to add functionality.

C. Carbonic Anhydrase Vectors and Host Cells

[00145] In order to produce a disclosed recombinant polypeptide, the DNA encoding the polypeptide can be chemically synthesized from published sequences or can be obtained directly from host cells harboring the gene (*e.g.*, by cDNA library screening or PCR amplification). In some embodiments, the carbonic anhydrase polynucleotide is included in an expression cassette and/or cloned into a suitable expression vector by standard molecular cloning techniques. Such expression cassettes or vectors contain sequences that assist initiation and termination of transcription (*e.g.*, promoters and terminators), and typically can also contain one or more selectable markers.

[00146] The expression cassette or vector is introduced into a suitable expression host cell, which then expresses the corresponding carbonic anhydrase polynucleotide. Suitable expression hosts are bacterial expression host genera including *Escherichia* (*e.g.*, *Escherichia coli*), *Pseudomonas* (*e.g.*, *P. fluorescens* or *P. stutzeri*), *Proteus* (*e.g.*, *Proteus mirabilis*), *Ralstonia* (*e.g.*, *Ralstonia eutropha*), *Streptomyces*, *Staphylococcus* (*e.g.*, *S. carnosus*), *Lactococcus* (*e.g.*, *L. lactis*), or *Bacillus* (*e.g.*, *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus licheniformis*, etc.). Also suitable are yeast expression hosts such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Yarrowia lipolytica*, *Hansenula polymorpha*, *Kluyveromyces lactis* or *Pichia pastoris*. Suitable fungal expression hosts include *Aspergillus niger*, *Chrysosporium lucknowense*, *Aspergillus* (*e.g.*, *A. oryzae*, *A. niger*, *A. nidulans*, etc.) or *Trichoderma reesei*. Also suited are mammalian expression hosts such as mouse (*e.g.*, NS0), Chinese Hamster Ovary (CHO) or Baby Hamster Kidney (BHK) cell lines. Other eukaryotic hosts such as insect cells or viral expression systems (*e.g.*, bacteriophages such as M13, T7 phage or Lambda, or viruses such as Baculovirus) are also suitable for producing the carbonic anhydrase polypeptide.

[00147] Promoters and/or signal sequences associated with secreted proteins in a particular host of interest are candidates for use in the heterologous production and secretion of thermostable carbonic anhydrases in that host or in other hosts. As an example, promoters from thermostable proteins, such as the *Bacillus licheniformis* thermostable amylase LAT promoter (pLAT), may be used. Alternatively, in filamentous fungal systems, the promoters that drive the genes for cellobiohydrolase I (*cbh1*), glucoamylase A (*glaA*), TAKA-amylase (*amyA*), xylanase (*exlA*), the *gpd*-promoter *cbh1*, *cbhll*, endoglucanase genes *eg1-eg5*, *Cel61B*, *Cel74A*, *gpd* promoter, *Pgk1*, *pki1*, *EF-1alpha*, *tef1*, *cDNA1* and *hex1* are suitable and can be derived from a number of different organisms (*e.g.*, *A. niger*, *T. reesei*, *A. oryzae*, *A. awamori*, *A. nidulans*).

[00148] In some embodiments, the carbonic anhydrase polynucleotide is recombinantly associated with a polynucleotide encoding a suitable homologous or heterologous signal sequence that leads to secretion of the recombinant carbonic anhydrase polypeptide into the extracellular (or periplasmic) space, thereby allowing direct detection of enzyme activity in the cell supernatant (or periplasmic space or lysate). Suitable signal sequences for *Escherichia coli*, other Gram negative bacteria and other organisms known in the art include those that drive expression of the HlyA, DsbA, Pbp, PhoA, PelB, OmpA, OmpT

or M13 phage Gill genes. For *Bacillus subtilis*, Gram-positive organisms and other organisms known in the art, suitable signal sequences further include those that drive expression of the AprE, NprB, Mpr, AmyA, AmyE, Blac, SacB, and for *S. cerevisiae* or other yeast, including the killer toxin, Bar1, Suc2, Mating factor alpha, Inu1A or Ggplp signal sequence. Signal sequences can be cleaved by a number of signal peptidases, thus removing them from the rest of the expressed protein. Additionally, signal sequences from thermostable proteins, including but not limited to, the *Bacillus licheniformis* LytD, NucB, BglC, hypothetical protein BLi01309 (cell wall-binding), YhcJ, and hypothetical protein BLi03260 may be used.

[00149] In some embodiments, the recombinant carbonic anhydrase polypeptide is expressed alone or as a fusion with other peptides, tags or proteins located at the N- or C-terminus (e.g., 6XHis, HA or FLAG tags). Suitable fusions include tags, peptides or proteins that facilitate affinity purification or detection (e.g., 6XHis, HA, chitin binding protein, thioredoxin or FLAG tags), as well as those that facilitate expression, secretion or processing of the target carbonic anhydrase. Suitable processing sites include enterokinase, STE13, Kex2 or other protease cleavage sites for cleavage in vivo or in vitro.

[00150] Carbonic anhydrase polynucleotides are introduced into expression host cells by a number of transformation methods including, but not limited to, electroporation, lipid-assisted transformation or transfection ("lipofection"), chemically mediated transfection (e.g., CaCl and/or CaP), lithium acetate-mediated transformation (e.g., of host-cell protoplasts), biolistic "gene gun" transformation, PEG-mediated transformation (e.g., of host-cell protoplasts), protoplast fusion (e.g., using bacterial or eukaryotic protoplasts), liposome-mediated transformation, *Agrobacterium tumefaciens*, adenovirus or other viral or phage transformation or transduction.

[00151] Alternatively, the recombinant carbonic anhydrase polypeptides are expressed intracellularly. Optionally, after intracellular expression of the enzyme variants, or secretion into the periplasmic space using signal sequences such as those mentioned above, a permeabilisation or lysis step can be used to release the recombinant carbonic anhydrase polypeptide into the supernatant. The disruption of the membrane barrier is effected by the use of mechanical means such as ultrasonic waves, pressure treatment (French press), cavitation, or by the use of membrane-digesting enzymes such as lysozyme or enzyme mixtures. As a further alternative, the polynucleotides encoding the recombinant carbonic anhydrase polypeptide are expressed using a suitable cell-free expression system. In cell-free systems, the polynucleotide of interest is typically transcribed with the assistance of a promoter, but ligation to form a circular expression vector is optional. In some embodiments, RNA is exogenously added or generated without transcription and translated in cell-free systems.

IV. Activities of Carbonic Anhydrases Polypeptides

[00152] The recombinant carbonic anhydrase polypeptides disclosed herein may have carbonic anhydrase activity over a broad range of pH conditions. In certain embodiments the disclosed recombinant carbonic anhydrase polypeptides have carbonic anhydrase activity when incubated for at

least 1 hour, at least 2.5 hours, at least 5 hours, at least 10 hours, at least 12 hours, at least 18 hours, at least 24 hours, at least one week, one month, six months, 12 months, 18 months or longer, at a pH of from about 8.0 to about 11, about 8.5 to about 10.5, or about 9.0 to about 10.0. Suitably, the recombinant carbonic anhydrase polypeptides have carbonic anhydrase activity at a pH of from about 8.5 to about 10.5. In some embodiments, the recombinant carbonic anhydrase polypeptides have carbonic anhydrase activity when incubated for at least 5 hours, at least 10 hours, at least 12 hours, at least 18 hours, at least 24 hours, or longer at a pH of about 6.0, 7.0, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, or 11.0. In further embodiments, the recombinant carbonic anhydrase polypeptides retain at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or higher carbonic anhydrase activity when incubated at a pH of from about 8.5 to about 10.5. It should be noted that the pH values described herein may vary by ± 0.2 . For example, a pH value of about 8.0 could vary from pH 7.8 to pH 8.2.

[00153] The recombinant carbonic anhydrase polypeptides disclosed herein may have carbonic anhydrase activity over a wide range of temperatures, *e.g.*, from about 0°C to about 80°C. In certain embodiments, the recombinant carbonic anhydrase polypeptides have carbonic anhydrase activity when incubated for at least 1 hour, at least 2.5 hours, at least 5 hours, at least 10 hours, at least 12 hours, at least 18 hours, at least 24 hours, at least one week, one month, six months, 12 months, 18 months or longer at a temperature of from about 0°C to about 80°C, about 0°C to about 70°C, about 0°C to about 60°C, about 0°C to about 50°C, about 0°C to about 45°C, about 0°C to about 40°C, about 0°C to about 35°C, about 0°C to about 30°C, about 0°C to about 25°C, about 0°C to about 20°C, about 0°C to about 15°C, about 0°C to about 10°C, or about 0°C to about 5°C. In some embodiments, the recombinant carbonic anhydrase polypeptides have carbonic anhydrase activity when incubated for at least 1 hour, at least 2.5 hours, at least 5 hours, at least 10 hours, at least 12 hours, at least 18 hours, at least 24 hours, at least one week, one month, six months, 12 months, 18 months or longer at a temperature of about 0°C, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C, 75°C or 80°C. It should be noted that the temperature values described herein may vary by $\pm 0.2^\circ\text{C}$. For example a temperature of about 50°C could vary from 49.8°C to 50.2°C.

[00154] In certain embodiments, the recombinant carbonic anhydrase polypeptides disclosed herein retain at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or more of carbonic anhydrase activity when incubated for at least 1 hour, at least 2.5 hours, at least 5 hours, at least 10 hours, at least 12 hours, at least 18 hours, at least 24 hours, at least one week, one month, six months, 12 months, 18 months or longer at a temperature of about 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C, 75°C or 80°C. In some embodiments, the incubation temperature is from about 40°C to about 80°C.

[00155] In further embodiments, the recombinant carbonic anhydrase polypeptides disclosed herein have carbonic anhydrase activity when incubated for at least 1 hour, at least 2 hours, at least 3 hours, at least 4 hours, at least 5 hours, at least 6 hours, at least 7 hours, at least 8 hours, at least 9 hours, at least 10 hours, at least 12 hours, at least 18 hours, at least 24 hours, at least one week, one month, six months, 12 months, 18 months or longer in the presence of bicarbonate (HCO_3^-). Suitably, the HCO_3^- concentration is achieved with 0.1 M, 0.25 M, 0.5 M, 1 M, 1.5 M, 2 M or a higher concentration of sodium bicarbonate (NaHCO_3), potassium bicarbonate (KHCO_3), or ammonium bicarbonate (NH_4CO_3). In related embodiments, the recombinant carbonic anhydrase polypeptides disclosed herein have increased or improved thermostability at increased ionic strengths. For example, the recombinant carbonic anhydrase polypeptides have a higher melting temperature at higher ionic strength conditions (e.g., at 1 M bicarbonate) as compared to at lower ionic strength conditions (e.g., at 0.8 M, 0.6 M, 0.4 M, 0.2 M, or 0.1 M bicarbonate).

[00156] The recombinant carbonic anhydrase polypeptides disclosed herein may have a wide range of melting temperatures depending upon pH, ionic strength conditions, and the presence of bicarbonate. In some embodiments, the recombinant carbonic anhydrase polypeptides have a melting temperature of at least about 50°C (e.g., at least about 56°C , 57°C , 58°C , 59°C , 60°C , 62°C , 64°C , 66°C , 68°C , 70°C , 72°C , 74°C , 76°C , 78°C , 80°C , 82°C , 84°C , 86°C , 88°C , 90°C , 92°C , 94°C , 96°C , 98°C , or even 100°C) at a pH of about 7.5 to about 11 (e.g., about 7.5 to about 10.5, about 8.0 to about 10.5, about 8.5 to about 10, about 9.0 to about 10.5, etc.). In certain embodiments, the recombinant carbonic anhydrase polypeptides have a melting temperature of about 56°C , 56.5°C , 57°C , 57.5°C , 58°C , 58.5°C , 59°C , 59.5°C , 60°C , 60.5°C , 61°C , 61.5°C , 62°C , 62.5°C , 63°C , 63.5°C , 64°C , 64.5°C , 65°C , 65.5°C , 66°C , 66.5°C , 67°C , 67.5°C , 68°C , 69°C , 70°C , 71°C , 72°C , 75°C , 78°C , 80°C , 82°C , 85°C , 90°C , or higher, at a pH of about 8.0, 8.5, 9.0, 9.5, 10.0, or 10.5. In some embodiments, the recombinant carbonic anhydrase polypeptides have a melting temperature of about 65°C , 69°C , 70°C , 71°C , 71.5°C , 72°C , 72.5°C , 73°C or higher, under high ionic strength conditions. Suitably, the high ionic strength condition is achieved by melting the recombinant carbonic anhydrase polypeptides in the presence of about 0.5 M, 1 M, 1.5 M, 2 M, or a higher concentration of sodium chloride (NaCl). In some embodiments, the recombinant carbonic anhydrase polypeptides have a melting temperature of about 65°C , 69°C , 70°C , 71°C , 71.5°C , 72°C , 72.5°C , 73°C or higher, in the presence of HCO_3^- . Suitably, the HCO_3^- concentration is achieved with 0.5 M, 1 M, 1.5 M, 2 M or a higher concentration of NaHCO_3 or KHCO_3 . In some embodiments, the recombinant carbonic anhydrase polypeptides have a melting temperature of about 70°C , 70.5°C , 71°C , 71.5°C , 72°C , 72.5°C , 73°C , 73.5°C , 74°C , 74.5°C , 75°C , 75.5°C , 76°C , 76.5°C , 77°C , 77.5°C , 80°C , 80.5°C , 81°C , 82°C , 83°C , 84°C , 85°C , 88°C , 90°C , 92°C , 95°C , 97°C , 100°C or higher, in the presence of HCO_3^- and under high ionic strength conditions. Suitably, the high ionic strength conditions is achieved by melting the recombinant carbonic anhydrase polypeptides in the presence of about 0.5 M, 1 M, 1.5 M, 2 M, or a higher concentration of NaCl , and in the presence of HCO_3^- , achieved with 0.5 M, 1 M, 1.5 M, 2 M or a higher concentration of NaHCO_3 or KHCO_3 . In

related embodiments, the recombinant carbonic anhydrase polypeptides have increased thermostability with increased ionic strengths. For example, the recombinant carbonic anhydrase polypeptides have increased or a higher melting temperature at a higher ionic strength condition (e.g., at 1 M bicarbonate) as compared to at a lower ionic strength condition (e.g., at 0.8, 0.6, 0.4, 0.2, or 0.1 M bicarbonate).

5 **[00157]** Example 1C shows that the recombinant Bgi CA1 polypeptide had carbonic anhydrase activity in carbon dioxide saturated water. In particular, the Bgi CA1 polypeptide was shown to have a specific activity of $4,023 \pm 387$ Wilbur-Anderson units/mg. Accordingly, in certain embodiments, any of the recombinant Bgi CA1 polypeptides disclosed herein convert carbon dioxide into a bicarbonate or carbonate product, or convert a biocarbonate or carbonate-containing material into CO_2 . In certain
10 embodiments, any of the recombinant Bgi CA1 polypeptides have improved specific activity and/or improved stability as compared to a native Bgi CA1.

[00158] Example 2C shows the recombinant Pvi CA1 polypeptide had carbonic anhydrase activity in carbon dioxide saturated water. In particular, the Pvi CA1 polypeptide was shown to have a specific activity of 937 ± 65 Wilbur-Anderson units/mg. Accordingly, in certain embodiments, any of the
15 recombinant Pvi CA1 polypeptides disclosed herein converts carbon dioxide into a bicarbonate or carbonate product, or convert a biocarbonate or carbonate-containing material into CO_2 . In certain embodiments, any of the recombinant Pvi CA1 polypeptides have improved specific activity and/or improved stability as compared to a native Pvi CA1.

[00159] Example 3C shows that the recombinant Ate CA1 polypeptide had carbonic anhydrase
20 activity in carbon dioxide saturated water. In particular, the Ate CA1 polypeptide was shown to have a specific activity of $1,327 \pm 285$ Wilbur-Anderson units/mg. Accordingly, in certain embodiments, any of the recombinant Ate CA1 polypeptides disclosed herein converts carbon dioxide into a bicarbonate or carbonate product, or converts a bicarbonate or a carbonate-containing material into CO_2 . In certain embodiments, any of the recombinant Ate CA1 polypeptides have improved specific activity and/or
25 improved stability as compared to a native Ate CA1.

[00160] Example 4C shows that the recombinant Spr CA1 polypeptide had carbonic anhydrase activity in carbon dioxide saturated water. In particular, the Spr CA1 polypeptide was shown to have a specific activity of $2,433 \pm 412$ Wilbur-Anderson units/mg. Accordingly, in certain embodiments, any of the recombinant Spr CA1 polypeptides disclosed herein convert carbon dioxide into a bicarbonate or
30 carbonate product, or convert a biocarbonate or carbonate-containing material into CO_2 . In certain embodiments, any of the recombinant Spr CA1 polypeptides have improved specific activity and/or improved stability as compared to a native Spr CA1.

[00161] Example 5C shows the recombinant Bag CA1 polypeptide had carbonic anhydrase activity in carbon dioxide saturated water. In particular, the Bag CA1 polypeptide was shown to have a specific
35 activity of 3715 ± 274 Wilbur-Anderson units/mg. Accordingly, in certain embodiments, any of the recombinant Bag CA1 polypeptides disclosed herein convert carbon dioxide into a bicarbonate or

carbonate product, or convert a bicarbonate or carbonate-containing material into CO₂. In certain embodiments, any of the recombinant Bag CA1 polypeptides have improved specific activity and/or improved stability as compared to a native Bag CA1.

[00162] Example 6B shows that the recombinant Vsp CA1 polypeptide had carbonic anhydrase activity in carbon dioxide saturated water. In particular, the Vsp CA1 polypeptide was shown to have a specific activity of 10,028+551 Wilbur-Anderson units/mg. Accordingly, in certain embodiments, any of the recombinant Vsp CA1 polypeptides disclosed herein convert carbon dioxide into a bicarbonate or carbonate product, or convert a bicarbonate or carbonate-containing material into CO₂. In certain embodiments, any of the recombinant Vsp CA1 polypeptides have improved specific activity and/or improved stability as compared to a native Vsp CA1.

[00163] Example 7B shows that the recombinant VspE CA1 polypeptide had carbonic anhydrase activity in carbon dioxide saturated water. In particular, the VspE CA1 polypeptide was shown to have a specific activity of 4621+431 Wilbur-Anderson units/mg. Accordingly, in certain embodiments, any of the recombinant VspE CA1 polypeptides disclosed herein convert carbon dioxide into a bicarbonate or carbonate product, or convert a biocarbonate or carbonate-containing material into CO₂. In certain embodiments, any of the recombinant VspE CA1 polypeptides have improved specific activity and/or improved stability as compared to a native VspE CA1.

[00164] Example 8B shows that the recombinant VspE CA2 polypeptide had carbonic anhydrase activity in carbon dioxide saturated water. In particular, the VspE CA2 polypeptide was shown to have a specific activity of 3189+112 Wilbur-Anderson units/mg. Accordingly, in certain embodiments, any of the recombinant VspE CA2 polypeptides disclosed herein convert carbon dioxide into a bicarbonate or carbonate product, or convert a biocarbonate or carbonate-containing material into CO₂. In certain embodiments, any of the recombinant VspE CA2 polypeptides have improved specific activity and/or improved stability as compared to a native VspE CA2.

V. Compositions Comprising a Recombinant Carbonic Anhydrase Polypeptide

[00165] In some embodiments, the present disclosure provides compositions comprising a recombinant carbonic anhydrase polypeptide (including variants or fragments, thereof) having carbonic anhydrase activity and methods for using such compositions in carbon dioxide extraction or sequestration applications. In some embodiments, the recombinant carbonic anhydrase polypeptide are selected from the group consisting of Bgi CA1, Pvi CA1, Ate CA1, Spr CA1, Bag CA1, Vsp CA1, VspE CA1, and VspE CA2.

[00166] Unless otherwise noted, all component or composition levels provided herein are made in reference to the active level of that component or composition, and are exclusive of impurities, for example, residual solvents or by-products, which may be present in commercially available sources.

[00167] In certain embodiments a recombinant carbonic anhydrase polypeptide of the present

disclosure is the major component of the composition, *e.g.*, a mono-component composition. Such compositions may further include an excipient, which, in the context of a mono-component composition, is to be understood as any auxiliary agent or compound used to formulate the composition and includes, without limitation, solvents (*e.g.*, water, inorganic salts, fillers, pigments, waxes), carriers, stabilizers, cross-linking agents, adhesives, preservatives, and buffers.

[00168] In certain embodiments, the composition may further include one or more enzymes other than the recombinant carbonic anhydrase polypeptide, including, without limitation, endo peptidases, aminopeptidases, amylases, carbohydrases, carboxypeptidases, catalases, cellulases, chitinases, cutinases, cyclodextrin glycosyltransferases, decarboxylases, deoxyribonucleases, esterases, alpha-galactosidases, beta-galactosidases, glucoamylases, alpha-glucosidases, beta-glucosidases, haloperoxidases, invertases, laccases, lipases, mannosidases, monooxygenases, nitrilases, oxidases, pectinolytic enzymes, peptidoglutaminases, peroxidases, phytases, polyphenoloxidases, proteolytic enzymes, ribonucleases, transglutaminases, xylanases, or combinations thereof.

[00169] In addition to the recombinant carbonic anhydrase polypeptides provided herein, one or more other suitable carbonic anhydrases can be included in the compositions of the present disclosure. Suitable carbonic anhydrases include, without limitation, alpha-carbonic anhydrases, beta-carbonic anhydrases, gamma-carbonic anhydrases, delta-carbonic anhydrases, epsilon-carbonic anhydrases, cytosolic carbonic anhydrases, mitochondrial carbonic anhydrases, secreted carbonic anhydrases, and membrane-associated carbonic anhydrases. Additionally, suitable carbonic anhydrases include, without limitation, those of animal, plant, fungal, bacterial, cyanobacterial, and diatomic origin. Chemically or genetically modified mutants are encompassed by the present disclosure.

[00170] In some embodiments, the polypeptides disclosed herein may comprise a modification. As used herein, "modification" describes various functional groups in a polypeptide that interact covalently, ionically, or by hydrophobic or hydrophilic association with various modifying agents. Covalent modifications to various polypeptides can be made by reaction of the enzyme with a hydrophobic agent, a hydrophilic agent, or an amphiphilic agent. These interactions add a hydrophobic, hydrophilic, or amphiphilic moiety to the enzyme. Various hydrophobic agents can be used, for example, a monoamine (*e.g.*, alkyl amine), an aldehyde (*e.g.*, pentanal, isobutanal, acetanal, hexanal, octanal, decanal), a quaternary ammonium salt, an alkyltrimethylammonium cation, an organic cation, a phosphonium cation, a pyridinium cation, an imidazolium cation, a viologen, a bis(triphenylphosphine)iminium metal complex, a bipyridyl metal complex, a phenanthroline- based metal complex, or a combination thereof. In various embodiments, the hydrophobic agent can be butyl amine, hexyl amine, octyl amine, decyl amine, dodecyl amine, pentanal, isobutanal, acetanal, hexanal, octanal, decanal, acetyltrimethylammonium bromide, sodium dodecyl sulfate, ammonium lauryl sulfate, triphenylphosphonium, hexadecylpyridinium, ethidium, methyl viologen, benzyl viologen, $[\text{Ru}(\text{bipyridine})_3]^{2+}$, $[\text{Fe}(\text{phenanthroline})_3]^{3+}$, or a combination thereof. In some embodiments, the

hydrophobic agent can be butyl amine, hexyl amine, octyl amine, decyl amine, dodecyl amine, pentanal, isobutanal, acetanal, hexanal, octanal, decanal, acetyltrimethylammonium bromide, sodium dodecyl sulfate, ammonium lauryl sulfate, triphenylphosphonium, hexadecylpyridinium, ethidium, methyl viologen, benzyl viologen, or a combination thereof. In certain embodiments, the polypeptides disclosed

herein may be covalently modified with an alkyl amine or a water soluble polymer, such as an unbranched or branched polyethylene glycol, an ethylene glycol/propylene glycol copolymer, carboxymethylcellulose, dextran, polyvinyl alcohol, and the like. Alkyl amines useful for covalent modification are butyl amine, hexyl amine, octyl amine, decyl amine, dodecyl amine, and the like. In some embodiments, the polypeptides disclosed herein may be modified by various surface active agents.

For example, non-ionic surface active agents can be N,N-bis(3-D-gluconamidopropyl)cholamide (BigCHAP), N,N-bis(3-D-gluconamidopropyl)deoxycholamide (DeoxyBigCHAP), a polyoxyethylene alcohol (*e.g.*, Brij35 and Brij 58 P), 2-cyclohexylmethyl- β -D-maltoside (Cymal-1), 2-cyclohexylethyl- β -D-maltoside (Cymal-2), cyclohexylpentyl- β -D-maltoside (Cymal-5), cyclohexylhexyl- β -D-maltoside (Cymal-6), decyl- β -D-maltopyranoside, n-dodecyl- β -D-maltoside, n-hexadecyl- β -D-maltoside, undecyl- β -D-maltoside, decyl- β -D-1-thiomaltopyranoside, octyl- β -D-thioglucopyranoside, digitonin, dimethydecylphosphine oxide, dodecyldimethylphosphine oxide, (octylphenoxy)polyethoxyethanol (IGEPAL[®] CA630), N-octanoyl-N-methylglucamine (MEGA-8), N-nonanoyl-N-methylglucamine (MEGA-9), N-decanoyl-N-methylglucamine (MEGA-10), a polyoxyethylene octyl phenol (Nonidet[®] P40-substitute), a polyoxyethylene-polyoxypropylene block co-polymer (Pluronic F-68), saponin, polyoxyethylene 9-lauryl ether (Thesit[®]), a polyoxyethylene octyl phenol (*e.g.*, Triton[®] X-100 and Triton[®] X-114), a polyoxyethylene derivative of sorbitan monolaurate (*e.g.*, TWEEN[®] 20, TWEEN[®] 40, and TWEEN[®] 80), N,N-dimethyldodecylamine-N-oxide, an alcohol ethoxylate (Synperonic A7), amidosulfobetaine-14, amidosulfobetaine-16, C7BzO, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS), 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonate (CHAPSO), (dodecyldimethylammonio)acetate (EMPIGEN[®] BB), 3-(N,N-dimethyloctylammonio)propanesulfonate, 3-(dodecylammonio)propanesulfonate, 3-(N,N-dimethylmyristylammonio)propanesulfonate, 3-(N,N-dimethylpalmitylammonio)propanesulfonate, 3-(N,N-dimethyloctadecylammonio)propanesulfonate, or a combination thereof.

[00171] The polypeptides disclosed herein may be modified at random positions within the molecule, or at predetermined positions within the molecule and may include one, two, three, or more attached chemical moieties. Methods for modifying polypeptides are known in the art, for example, EP 0401384 and Malik et al., Exp. Hematol. 20:1028-1035, 1992 (reporting PEGylation of GM-CSF using tresyl chloride).

[00172] In certain embodiments, the polypeptides disclosed herein may be immobilized. In certain embodiments, methods of protein engineering can be applied to the carbonic anhydrase polypeptide herein such that one or more sites, which are not previously present in the polypeptide, can be engineered into the polypeptide to facilitate immobilization; or alternatively, protein engineering methods can be

used to alter certain of the residues in the polypeptide such that the polypeptide becomes more amenable to or suitable for immobilization. An immobilized polypeptide can contain two types of functions: (1) non-catalytic functions that are designed to aid separation (*e.g.*, isolation of polypeptide from the application environment, reuse of the polypeptide, or control of the process); and (2) catalytic functions that are designed to convert the target compounds (or substrates) within the time and space desired (Cao, Carrier-bound Immobilized Enzymes: Principles, *Applications and Design*, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 2005). When a polypeptide is immobilized, it is made insoluble to the target compounds (*e.g.*, substrates) it aids in converting, and to the solvents used. An immobilized polypeptide product can be separated from the application environment in order to facilitate its reuse, or to reduce the amount of polypeptide needed, or to use the polypeptide in a process where substrate is continuously delivered and product is continuously removed from proximity to the polypeptide. This approach may, for example, contribute to a reduced cost of polypeptide expenditure. Furthermore, polypeptides are often stabilized by immobilization. A process involving immobilized polypeptide is often continuous, which facilitates easy process control. The immobilized polypeptide can be retained as a heterogeneous catalyst by mechanical means, or by inclusion in a definite space. The latter can be done by microencapsulation, *e.g.*, in semi permeable membranes or by inclusion in UF systems using, *e.g.*, hollow fiber modules, etc. Immobilization on porous carriers is also commonly used. This includes binding of the polypeptide to the carrier, *e.g.*, by adsorption, complex/ionic/covalent binding, or by simple absorption of soluble polypeptide on the carrier and subsequent removal of solvent. Cross-linking of the polypeptide can also be used as a means of immobilization. Additionally, a polypeptide may be immobilized by inclusion into a carrier (Buchholz et al., *Biocatalysts and Enzyme Technology*, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 2005).

[00173] There are a variety of methods for polypeptide immobilization, including without limitation, carrier-binding, cross-linking, and entrapping. Carrier-binding is the binding of polypeptides to water-insoluble carriers. Cross-linking is the intermolecular cross-linking of polypeptides by bifunctional or multifunctional reagents. Entrapping is incorporating polypeptides into the lattices of a semipermeable material. Additional methods of immobilizing polypeptides include, without limitation, spraying of the polypeptide together with a liquid medium comprising a polyfunctional amine and a liquid medium comprising a cross-linking agent onto a particulate porous carrier as described in WO 2007/036235, linking of the polypeptide with a cross-linking agent (*e.g.*, glutaraldehyde) to an ovalbumin layer, which in turn adheres to an adhesive layer on a polymeric support as described in WO 2005/114417, coupling of the polypeptide to a silica carrier as described in U.S. Patent 5,776,741, or to a silane, or a CNBr activated carrier surface such as glass, or co-polymerization of the polypeptide with methacrylate on polymer beads as described in Bhattacharya et al., *Biotechnol. Appl. Biochem.* 38: 111-117, 2003. The particular method of polypeptide immobilization used in connection with the compositions and methods described herein is flexible, so long as the immobilization material (1) immobilizes the polypeptide, and/or in some embodiments, (2) stabilizes the polypeptide. In various embodiments, the immobilization

material is also permeable to a compound smaller than the polypeptide. A polypeptide is adsorbed to an immobilization material when it adheres to the surface of the material by chemical or physical interactions. Further, a polypeptide is immobilized by entrapment when the polypeptide is contained within the immobilization material, whether or not within a pocket of the material.

- 5 **[00174]** In certain embodiments, the immobilization material is permeable to various compounds that are smaller than a polypeptide. Such permeability allows the movement of a substrate compound through the material, such that the substrate compound can contact the polypeptide. The immobilization material can be prepared in a manner such that it contains internal pores, micellar pockets, channels, openings or a combination thereof, which allow the movement of the substrate compound throughout the
- 10 immobilization material, but which constrain the polypeptide to substantially the same space within the immobilization material. Such constraint allows the polypeptide to retain its function or catalytic activity, or even to increase its catalytic activity and/or stability. In various embodiments, the polypeptide is confined to a space that is substantially the same size and shape as the polypeptide, wherein the polypeptide retains substantially all of its function or catalytic activity. The pores, micellar pockets,
- 15 channels, or openings have physical dimensions that satisfy the above requirements, depending on the size and shape of the specific polypeptide to be immobilized.

- [00175]** In certain embodiments, the immobilization material has a micellar or inverted micellar structure. Generally, the molecules making up a micelle are amphipathic, meaning they contain a polar, hydrophilic group and a nonpolar, hydrophobic group. The molecules can aggregate to form a micelle,
- 20 where the polar groups are on the surface of the aggregate and the hydrocarbon, nonpolar groups are sequestered inside the aggregate. Inverted micelles have the opposite orientation of polar groups and nonpolar groups. The amphipathic molecules making up the aggregate can be arranged in a variety of ways so long as the polar groups are in proximity to each other and the nonpolar groups are in proximity to each other. Also, the molecules can form a bilayer with the nonpolar groups pointing toward each
- 25 other and the polar groups pointing away from each other. Alternatively, a bilayer can form wherein the polar groups can point toward each other in the bilayer, while the nonpolar groups point away from each other. Examples of micellar or inverted micellar immobilization material include, without limitation, a modified perfluoro sulfonic acid-PTFE copolymer (or modified perfluorinated ion exchange polymer) (modified NAFION[®] or modified FLEMION[®]) membrane; hydrophobically modified polysaccharides,
- 30 such as chitosan, cellulose, chitin, starch, amylose, alginate, glycogen, and combinations thereof; polycationic polymers, such as hydrophobically modified chitosan; and polyanionic polymers, such as hydrophobically modified alginate. Other examples of immobilization material include, without limitation, modified polysulfone, modified polycarbonate, modified poly(vinylbenzyl chloride), modified polysiloxanes, and modified polysulfone-graft-polyethylene glycol. The above immobilization materials
- 35 are described in detail in WO 2010/037109 and U.S. Patent Application Publication No. US 2010/0209968.

[00176] Methods of encapsulating or immobilizing a polypeptide in any of the above immobilization materials are known in the art. For example, WO 2010/037109 describes methods of encapsulating a polypeptide in polysulfone, and immobilizing a polypeptide in alginate, poly(vinylbenzyl chloride), and polysulfone-graft-polyethylene glycol.

5 **[00177]** In certain embodiments, the immobilization material may include, without limitation, a polymer, a membrane including liquid membranes, a matrix, a wafer, a solid support, or a micro-particle. In some embodiments, the immobilization material may be selected from beads, fabrics, fibers, hollow fibers, membranes, particulates, porous surfaces, rods, structured packing, and tubes. Further examples of suitable immobilization material include, without limitation, alumina, bentonite, biopolymers, calcium carbonate, calcium phosphate gel, carbon, cellulose, ceramic supports, clay, collagen, glass,
10 hydroxyapatite, ion-exchange resins, kaolin, nylon, phenolic polymers, polyaminostyrene, polyacrylamide, polypropylene, polymerhydrogels, sephadex, sepharose, silica gel, precipitated silica, and TEFLON®-brand PTFE.

[00178] Once a polypeptide has been immobilized within the immobilization material, the
15 immobilized polypeptide can be deposited on a support. The substrate can be a material that provides the desired mechanical support necessary for the selected use. For example, the support may be a filter, a wire mesh, porous polymer, organic and inorganic membrane, and the like when the immobilized polypeptide is used as a catalyst for a chemical transformation.

[00179] In some embodiments, compositions comprising immobilized polypeptides can be core
20 particles. A core particle is any particle that provides a support for the immobilized polypeptide layer. A core particle can be spray-dried. The core particle can be, for example, a polymer particle, a carbon particle, a zeolite particle, a metal particle, a ceramic particle, a metal oxide particle, a silica particle, or a combination thereof. In some embodiments, the core particle is an inert core particle. In some
25 embodiments, the core particle is not a polymer particle. Suitably, core particles do not adversely affect the stability of the polypeptide or a chemical transformation involving the polypeptide. Methods for preparing core particles are known in the art. For example, WO 2010/037109 describes methods for coating a core particle with a mixture containing the polypeptide and immobilization material.

[00180] In some embodiments, the polypeptides disclosed herein may be stabilized by any method known in the art *e.g.*, by stabilizing the polypeptide in the composition by adding an antioxidant or
30 reducing agent to limit oxidation of the polypeptide or by adding compounds that trap/inactivate the compounds that damage the enzyme or it may be stabilized by adding polymers such as PVP, PVA, PEG, sugars, oligomers, polysaccharides or other suitable polymers known to be beneficial to the stability of polypeptides in solid or liquid compositions. A preservative, such as Proxel™, can also be added to extend shelf life or performance of the composition.

35 **[00181]** In certain embodiments, the compositions of the present disclosure can be used for capturing carbon dioxide.

[00182] The compositions of the present disclosure may be prepared in accordance with methods known in the art, which may be in the form of a liquid or a solid composition. For example, the composition may be formulated using methods known to the art of formulating technical enzymes and/or pharmaceutical products, *e.g.*, into coated or uncoated granules or micro-granules. The recombinant carbonic anhydrase polypeptides of the present disclosure may thus be provided in the form of a granule, such as a non-dusting granule, a liquid, in particular a stabilized liquid, a slurry, or a protected polypeptide.

VI. Uses of Carbonic Anhydrase Polypeptides

[00183] The recombinant carbonic anhydrase polypeptides disclosed herein are thermostable and can catalyze the interconversion of carbon dioxide (CO₂) and bicarbonate. In certain embodiments, a recombinant carbonic anhydrase polypeptide of the present disclosure can be used to hydrate CO₂ in the form of bicarbonate and a proton, which in turn, may be converted to carbonate and/or a mixture of bicarbonate and carbonate at an elevated pH. In some embodiments, a recombinant carbonic anhydrase polypeptide of the present disclosure can be used to release CO₂ from bicarbonate under conditions that favor the dehydration of bicarbonate. In some embodiments, the recombinant carbonic anhydrase polypeptide are selected from the group consisting of Bgi CA1, Pvi CA1, Ate CA1, Spr CA1, Bag CA1, Vsp CA1, VspE CA1, and VspE CA2.

[00184] Accordingly, in certain embodiments, the present disclosure provides methods for extracting CO₂ from a CO₂-containing medium, such as a gas, a liquid, or multiphase mixture, by contacting the CO₂-containing medium with any of the disclosed compositions comprising a recombinant carbonic anhydrase polypeptide to yield a medium reduced in CO₂. In some embodiments, the CO₂ is extracted to another medium such as a gas or liquid separated from the first medium, but the extraction may also be the conversion of CO₂ to bicarbonate or of bicarbonate to CO₂, within the same medium. The carbonic anhydrase polypeptides disclosed herein are useful where the temperature of the CO₂-containing medium is high (*e.g.*, where commercially available carbonic anhydrases, such as CA-I or CA-II isolated from bovine erythrocytes are not stable).

[00185] In certain embodiments, the methods disclosed herein may be used for extracting CO₂ from CO₂ emission streams, *e.g.*, from carbon-based or hydrocarbon-based combustion in electric generation power plants, or from flue gas stacks from such plants, industrial furnaces, stoves, ovens, or fireplaces or from airplane or car exhausts. The disclosed methods may also be used to remove CO₂ in the preparation of industrial gases, including, without limitation, acetylene (C₂H₂), carbon monoxide (CO), chlorine (Cl₂), hydrogen (H₂), methane (CH₄), nitrous oxide (N₂O), propane (C₃H₈), sulfur dioxide (SO₂), argon (Ar), nitrogen (N₂), and oxygen (O₂). The disclosed methods may also be used to remove CO₂ from a raw natural gas during the processing to natural gas. Removal of CO₂ from the raw natural gas will serve to enrich the methane (CH₄) content in the natural gas, thereby increasing the thermal units/m³. Raw natural gas is generally obtained from oil wells, gas wells, and condensate wells. Natural gas contains between 3

and 10% CO₂ when obtained from geological natural gas reservoirs by conventional methods.

[00186] In resemblance to the methane enrichment of natural gases, the disclosed methods can also be used to enrich the methane content in biogases. Biogases contain a considerable degree of CO₂, since the bacteria used in the fermentation process produce methane (60-70%) and CO₂ (30-40%). Biogas
5 production may be performed using mesophilic or thermophilic microorganisms. The process temperatures for mesophilic strains are approximately between 25°C and 40°C. In this temperature range a carbonic anhydrase may be utilized that is of bovine origin since the enzyme is not required to be thermostable. However, a carbonic anhydrase that tolerates higher temperatures, such as the disclosed recombinant carbonic anhydrase polypeptides, will offer improved robustness and fidelity in actual use
10 and storage related to biogas processes utilizing mesophilic strains. Thermophilic strains allow the fermentation to occur at elevated temperatures, *e.g.*, from 40°C to 75°C. In such processes a heat-stable carbonic anhydrase is particularly useful in methods for removing CO₂ from the methane. In certain embodiments, the methods of present disclosure may be used in the production of syngas by removing the CO₂ generated by the gasification of a carbon containing fuel (*e.g.*, methane or natural gas) thereby
15 enriching the CO and H₂ content of the syngas.

[00187] In certain aspects of the methods disclosed herein, the CO₂ extraction from a CO₂-containing medium can be performed in enzyme-based bioreactors. Before the CO₂-containing medium is processed in a bioreactor, it may be purified to free it from contaminants which may disturb the enzymatic reaction or interfere with bioreactor functionality in other ways, *e.g.*, by clotting outlets or membranes.

20 Gasses/multiphase mixtures emitted from combustion processes, *e.g.*, flue gases or exhausts, may be cleared of ash, particles, NO_x and/or SO₂, before the gas/multiphase mixture is passed into the bioreactor. The raw natural gas from different regions may have different compositions and separation requirements. Oil, condensate, water, and natural gas liquids, if present in the raw natural gas, may also be removed prior to the extraction of CO₂ in an enzyme based bioreactor. The CO₂ from the raw natural gas may be
25 extracted in the same process as the sulfur removal, or it may be extracted in a completely separate process. If the gas at this point exceeds the thermostability range of the carbonic anhydrase polypeptides of the present disclosure, some degree of cooling may be needed. Additionally, the reaction temperature may be between 40°C and 75°C. However, due to the thermostability of the disclosed Carbonic anhydrase polypeptides, the need for cooling can be at least 5°C less than if a CA-I or CA-II isolated
30 from bovine erythrocytes is applied in the bioreactor.

[00188] One example of a suitable type of bioreactor that may be used with any of the compositions or methods disclosed herein is based on a process in which a mixed gas stream (*e.g.*, containing oxygen, nitrogen and carbon dioxide) contacts an enzyme such as a carbonic anhydrase, at a gas-liquid interface to catalyze the conversion of carbon dioxide contained in the gas to bicarbonate or carbonate. The gas-
35 liquid interface in such a bioreactor can for example be provided by an enzyme based hollow fiber membrane bioreactor (HFMB). An example of HFMB is a hollow fiber contained liquid membrane

(HFCLM) as described by Majumdar et al., *AIChE* 1135-1145, 1988. CLMs are made by sandwiching a core liquid between two polymer membranes. The core liquid is suitably continuously re-supplied through a reservoir of liquid membrane solvent. An alternative type of enzyme based CLM permeator useful in a bioreactor is described in Cowan et al., *Ann. NY Acad. Sci.* 984: 453-469, 2003. For example, the bioreactor may contain a liquid membrane constructed by sandwiching a carbonic anhydrase-containing phosphate buffered solution between two hydrophobic, microporous, polypropylene membranes (*e.g.*, Celgard PP-2400). The liquid membrane fluid volume can be maintained by hydrostatic fluid addition from a reservoir, ensuring a constant liquid membrane thickness and prevents separation between the polymer membrane and the metal support. One side of the CLM (the feed membrane) may be contacted with a CO₂-containing feed gas stream, and the other side of the CLM (the sweep membrane) may be in contact with a CO₂-free sweep gas stream, for example argon. In this bioreactor CO₂ from the feed gas stream is converted to bicarbonate in the liquid phase and then returned as CO₂ to the sweep gas stream from where it can be stored in the form of compressed CO₂. The entire process is catalyzed by the carbonic anhydrase. Alternative CLM permators may be composed of hollow-fiber membrane mats, *e.g.*, Celgard X40-200 or X30-240 instead of hydrophobic, microporous, polypropylene membranes. The hollow-fiber permeator can be arranged into different designs. In one design the permeator is arranged much like a heat exchanger and consists of multiple sets of hollow fiber feed fibers and hollow fiber sweep fibers arranged orthogonally while a carrier fluid fills the space between the feed and sweep fiber bundles (see for example Majumdar et al., *AIChE* 1135-1145, 1988). Another design is a spiral wound hollow fiber design that can operate in either co-current or counter-current mode. WO 04/104160 describes these and other hollow-fiber permator designs in more detail. WO 04/104160 also describes the use of a phosphate buffer as the membrane liquid. When carbonic anhydrase is added to the membrane liquid it was either dissolved in phosphate buffer or in 1 M NaHCO₃. The above type of bioreactor is described in detail in WO 2010/014774 and WO 2010/151787.

[00189] Another example of a suitable type of bioreactor that may be used with any of the compositions or methods disclosed herein is based on a process in which a gas phase or multiphase mixture, is contacted with a liquid phase under conditions where the CO₂ in the gas phase is absorbed by the liquid phase where it is converted into bicarbonate by a carbonic anhydrase. The bicarbonate enriched liquid is removed from the reactor by a continuous flow, to ensure that the equilibrium between CO₂ and bicarbonate is shifted towards continuous conversion of CO₂. The gas phase dissolution into the liquid phase is dependent on the surface contact area between the gas and liquid. A large contact area can either be achieved by passing liquid and CO₂-containing gas through a packed column or by bubbling the CO₂-containing gas through the liquid generating an elevated pressure in the reaction chamber. For example, packed columns can be composed of packings such as raschig rings, berl saddles, intalox metal, intalox saddles, pall rings. The packing materials may be a polymer such as nylon, polystyrene a polyethylene, a ceramic such as silica, or a metal such as aluminum. In both reactor types the liquid is continuously exchanged, hence carbonic anhydrase must be retained in the reactor by various means. In the packed

columns the carbonic anhydrase can be immobilized on the packing material. In the “bubbling” reactors the carbonic anhydrase can be entrapped in a porous substrate, for example, an insoluble gel particle such as silica, alginate, alginate/chitosane, alginate/carboxymethylcellulose, or the carbonic anhydrase can be immobilized on a solid packing (as in the packed columns) in suspension in the liquid, or the carbonic anhydrase can be chemically linked in an albumin or PEG network. When the reactors are in operation an aqueous or organic solvent enters the reactor at one end, preferably the top, and flows to the other end, preferably the bottom, and the CO₂-containing gas stream (feed gas) enters the reactor at one end, preferably at the opposite end of the solvent (the bottom) and the gas passes through the liquid and exits through a gas outlet at the opposite end (preferably, the top of the reactor). The solvent/liquid that exits the reactor is enriched in bicarbonate and the exit gas is reduced in the CO₂ content compared to the feed gas. The bicarbonate containing solution may be processed in subsequent reactions for example to generate pure CO₂ or carbonate precipitates such as CaCO₃. The exit gas may also be subjected to further rounds of CO₂ extraction. The above type of bioreactor is described in detail in U.S. Patent 6,524,843, WO 2004/007058, and WO 2010//151787.

[00190] Another suitable type of bioreactor that may be used with any of the compositions or methods disclosed herein utilizes a gas diffusion membrane, such that gaseous CO₂, or CO₂ from a multiphase mixture is diffused into a capturing liquid by allowing the gaseous CO₂ to pass through a gas diffusion membrane. The CO₂ may pass into the liquid by diffusion (pressure aided) or the transfer may be aided by a carbonic anhydrase, such as the carbonic anhydrase polypeptides disclosed herein, immobilized on the diffusion membrane, *e.g.*, by cross-linking or by affixing a gel or polymer matrix containing the carbonic anhydrase onto the diffusion membrane. Since the carbonic anhydrase reacts specifically with dissolved CO₂, it favors the movement of gaseous CO₂ into the fluid by accelerating the reaction of the dissolved CO₂ and water to form carbonic acid, thereby removing CO₂ rapidly and allowing the dissolution of CO₂ from the gas from the feed stream into the water to a greater extent than it would otherwise. The gas diffusion membrane may have a high surface area to facilitate a large flow of the gaseous CO₂ through the membrane. Suitable membranes include, without limitation, a polypropylene gas exchange membrane, ePTFE (GORE-TEX®), NAFION® membranes, zeolites, chytosan, polyvinylpyrrolindine, cellulose acetate, and immobilized liquid membranes. The CO₂ /bicarbonate rich fluid that emerges from the gas diffusion membrane is passed by a matrix that contains carbonic anhydrase. The matrix may be contained in a chamber which is separate from the chamber containing the diffusion membrane. Examples of suitable matrixes include, without limitation, beads, fabrics, fibers, membranes, particulates, porous surfaces, rods, and tubes. Specific examples of suitable matrixes include alumina, bentonite, biopolymers, calcium carbonate, calcium phosphate gel, carbon, cellulose, ceramic supports, clay, collagen, glass, hydroxyapatite, ion-exchange resins, kaolin, nylon, phenolic polymers, polyaminostyrene, polyacrylamide, polypropylene, polymerhydrogels, sephadex, sepharose, silica gel, and TEFLON®-brand PTFE. The carbonic anhydrase may be immobilized to the matrix or entrapped within it. Once the CO₂ is passed into the liquid, an equilibrium between carbonic

acid, bicarbonate and carbonate ions will be established, which process is catalyzed by carbonic anhydrase. Base (*e.g.*, OH⁻ ions) can then be added to shift the equilibrium to favor the formation of carbonate ions. In the final step, a mineral ion is added to a solution to precipitate carbonate salts. Alternatively, no base is added, thereby predominantly generating bicarbonate ion which can be concentrated using an ion-exchange resin or membrane. The bicarbonate can then be precipitated using sodium, magnesium, or calcium ions. The above type of bioreactor is described in detail in U.S. Patent 7,132,090.

[00191] A further suitable type of bioreactor that may be used with any of the compositions or methods disclosed herein includes a partition membrane and a phase-conversion membrane that selectively absorbs a desired component gas from a mixed stream and converts it into a second phase thereby isolating and purifying the desired component. The partition membranes include, for example, hollow fibers. These hollow fibers can be functionalized to accept covalent or other types of bonding with materials that can act as a bridge to yet other materials, here, for example an enzyme such as a carbonic anhydrase. Additionally, the phase-conversion membrane can be composed of aqueous solvents, protic solvents, aprotic solvents, hydrocarbons, aromatic hydrocarbons, ionic liquids and supercritical fluids, such as supercritical carbon dioxide or supercritical water. Moreover, the phase-conversion membrane can chemically convert the gas to an ionic species soluble in an aqueous medium by way of a phase-conversion catalyst, suitably, an enzyme such as a carbonic anhydrase. The above type of bioreactor is described in detail in WO 2004/104160.

[00192] Still another suitable type of bioreactor that may be used with any of the compositions or methods disclosed herein includes a resin-wafer electrodeionization (RW-EDI) for removing the carbon dioxide. The reactor includes cathode and anode electrodes separated by a plurality of porous solid ion exchange resin wafers, which when in use are filled with an aqueous fluid. The plurality of wafers can include one or more basic wafers arranged in a stack between the cathode and the anode. The wafers, anode, and cathode can be interleaved with ion exchange membranes. The basic wafers may contain a porous basic ion exchange medium, and may be adapted to (a) introduce a CO₂-containing gas into an aqueous fluid within the basic ion exchange medium to convert CO₂ from the gas into bicarbonate ion, and (b) vent a CO₂-depleted gas therefrom. In use, CO₂ is converted to bicarbonate in the fluid within the wafer under the basic conditions of the basic ion exchange medium. The bicarbonate-containing fluid can then be transported out of the reactor as a concentrated bicarbonate ion solution. The basic ion exchange medium of each wafer may contain a carbonic anhydrase polypeptide to facilitate conversion of gaseous CO₂ into bicarbonate ion. Additionally, the carbonic anhydrase polypeptide can be chemically or biochemically bound to the basic ion exchange medium to facilitate conversion of gaseous CO₂ into bicarbonate ion. The basic pH value of the basic ion exchange medium can be maintained by applying an electric potential appropriate to achieve the desired current (*e.g.*, commonly about 1 to 6 volts per cell pair) across the cathode and anode to provide a driving force for transport of protons (H⁺) toward the cathode and hydroxyl ions (OH⁻) toward the anode through the wafers and ion exchange membranes. The

transport of protons and hydroxyl ions may be balanced with the flow of other cations and anions present in the fluid within the ion exchange media of the wafer to maintain the pH in each wafer or portion in its basic state. The above type of bioreactor is described in detail in WO 2010/138792.

[00193] The enzyme based bioreactors described above also find more unconventional applications such as in pilot cockpits, submarine vessels, aquatic gear, safety and firefighting gear and astronaut's space suits to keep breathing air free of toxic CO₂ levels. Other applications are to remove CO₂ from confined spaces, such as to reduce hazardous CO₂ levels from inside breweries and enclosed buildings carrying out fermentation, and from CO₂ sensitive environments like museums and libraries, to prevent excessive CO₂ from causing acid damage to books and artwork.

[00194] The recombinant carbonic anhydrase polypeptides of the present disclosure may be used as an independent CO₂ extraction catalyst or may alternatively be combined with conventional CO₂ extraction technologies such as chemical absorption via amine-based solvents or aqueous ammonia or physical solvents such as SELEXOL™ (Union Carbide) or polyethylene glycol ethers. Such combinations may either be applied in the bioreactors described above or may be applied to already existing CO₂ scrubbing facilities based on more conventional techniques.

[00195] The recombinant carbonic anhydrase polypeptides disclosed herein also find use in processes for recycling carbon dioxide emissions from a fossil-fuel power plant into useful carbonated species. For example, carbon dioxide emissions may be recycled from a power plant that utilizes either coal or natural gas. In the case of coal, the fuel is burned in a combustion chamber; the heat is used to produce steam from water in a heat recovery steam generator system. The steam propels turbines and alternators producing electric power. The flue gas exiting the combustion chamber is treated to remove ash, NO_x and/or SO_x. Rather than exhausting the gas by a stack, the gas is sent to additional heat exchangers and energy recovery systems to cool it down to an adequate temperature for the biological process. Energy is produced by this step. The cooled gas is then treated in a gas treatment unit to remove additional contaminants that may be harmful to the biological process, and finally, CO₂ is removed by a bioreactor that contains a free and/or immobilized polypeptide, such as a carbonic anhydrase, that converts CO₂ to bicarbonate, and the low CO₂ gas is blown to the atmosphere. In the case of natural gas, the fuel is burned directly in the turbine, and the intermediary step of steam production is not present in the main power production stage, although it may be used in subsequent heat recovery stages. This process may also be integrated with a SO_x treatment unit. The above process for recycling carbon dioxide emissions from a fossil-fuel power plant is described in detail in U.S. Patent 7,596,952.

[00196] Additionally, the recombinant carbonic anhydrase polypeptides disclosed herein find use in processes for capturing carbon dioxide from a CO₂-containing gas using carbonates and biocatalysts or micro-particles containing biocatalysts. For example, this processes includes contacting a CO₂-containing gas with an absorption mixture that includes water, biocatalysts, such as carbonic anhydrases, and a carbonate compound to enable dissolution and transformation of CO₂ into bicarbonate and

hydrogen ions, thereby producing a CO₂-depleted gas and an ion-rich solution; and subjecting the ion-rich solution to desorption where the carbonate compound promotes release of the bicarbonate ions from the ion-rich solution producing a CO₂ gas stream and an ion-depleted solution. In certain embodiments, the biocatalyst may be comprised in a micro-particle, which can be provided at a concentration sufficient for the absorption mixture flows through the packed bioreactor and are carried with a liquid solution to promote dissolution and transformation of CO₂ into bicarbonate and hydrogen ions. Additionally, the carbonate compound may be of a type and may be added in sufficient quantities to promote precipitation of a bicarbonate species during absorption. The precipitates may be part of the ion-rich solution that is sent for desorption or treated separately for conversion into CO₂ gas. Additionally, the carbonate compound may be chosen to allow the precipitation of bicarbonate species, such as KHCO₃, NaHCO₃, or NH₄HCO₃. As such, the carbonate compound may include, without limitation, potassium carbonate, sodium carbonate, ammonium carbonate, promoted potassium carbonate solutions and promoted sodium carbonate solutions or promoted ammonium carbonate or mixtures thereof. The above process for capturing carbon dioxide from a CO₂-containing gas is described in detail in WO 2011/014956 and WO 2011/01457.

[00197] The recombinant carbonic anhydrase polypeptides disclosed herein also find use in systems for accelerating the rate of absorption and reaction of CO₂ into the aqueous phase of a carbonate solution to form bicarbonate. In particular, the systems may be used to hydrate carbon dioxide gas in a gas stream to form bicarbonate ions. The systems may utilize a variety of bioreactors, including without limitation, a packed bed, a fluidized bed, a continuous stirred tank, or any other bioreactor disclosed herein. When a packed or fluidized bed bioreactor is used, the gas and liquid streams entering the bioreactor can be in a co-current or counter current configuration. For example, in a co-current system, the gas and liquid streams may enter the bioreactor in the form of microbubbles of gas in the liquid stream. Further, the packing of the bioreactors may be packing material containing an immobilized carbonic anhydrase. For example, the immobilized carbonic anhydrase may be coated on the packing material. In some embodiments, the packing material has a high surface area. Further, the configuration in the bioreactor may be similar to a tray style distillation column wherein the packing material includes a membrane comprising the immobilized carbonic anhydrase is oriented to maximize the surface contact with the gas and liquid streams (*e.g.*, by folding the membrane back on itself in a serpentine configuration).

[00198] In one example of such a system, a two unit continuous flow system can be used to hydrate CO₂ gas to form bicarbonate ions in a CO₂ absorber and dehydrate the bicarbonate ions to CO₂, water, and carbonate ions in a CO₂ desorber. In some instances, the units may have a packed tower design. A CO₂ gas stream enters the bottom of the absorber, and a liquid stream enters the top portion of the absorber. The liquid stream may be distributed over the top of the packing in the middle portion of the absorber by a distributor. The liquid stream wets the surfaces of the packing and flows downward through the absorber while the CO₂ gas stream flows upward through the interstices in the packing countercurrent to the flow of the liquid. The packing can provide an area of contact between the liquid

and gas phases, and includes a carbonic anhydrase immobilized on its outer surface. The CO₂ in the gas stream is absorbed by the liquid, and the treated gas stream leaves the top of the absorber. The liquid is enriched in CO₂ as it flows down the column, bicarbonate is formed, and the treated liquid stream leaves the bottom of the absorber. The treated liquid stream can then be pumped to a top portion of the desorber, and may be distributed by a distributor over packing having the immobilized carbonic anhydrase. The bicarbonate within the liquid stream can then be converted to carbon dioxide, water, and carbonate. Reaction rates of this reaction to produce CO₂ may be increased by adding heat and by increasing the rate of removal of CO₂ from the desorber by operating at below atmospheric pressure. The water and carbonate can be recycled and combined with the liquid stream entering the absorber, and the carbon dioxide may leave the top of the desorber as a gas stream and can be further processed as desired. Alternatively, the absorber may have a carbonic anhydrase immobilized on standard bioreactor packing materials (such as Berl saddle, Intalox saddle, Raschig ring or Pall ring packings commonly used in packed towers) and can be contacted with a microbubble CO₂ gas and an aqueous carbonate solution to allow for increased surface area between the gas and liquid for transport of the CO₂ gas into the aqueous carbonate solution.

[00199] In certain embodiments, the system may include a bioreactor having a membrane wherein a gas stream containing CO₂ is in contact with a first surface of the membrane and an aqueous carbonate stream is on a second surface of the membrane. The membrane may be permeable to at least the CO₂ gas, but is either impermeable to the aqueous carbonate stream or the first surface is impermeable to the stream. The membrane may also support an immobilized carbonic anhydrase. The CO₂ gas in the gas stream can interact with the immobilized carbonic anhydrase and the stream, and then be converted to bicarbonate. The bicarbonate can then be absorbed by the stream in contact with the immobilized enzyme. The membrane material may be a polysaccharide, an ion exchange resin, a treated silicon oxide, a porous metal structure, a carbon rod or tube, a graphite fiber, a silica bead, a cellulose membrane, or a gel matrix (*e.g.*, a polyacrylamide gel, a poly(acryloyl morpholine) gel, a nylon mesh and the like). The desorber may also have a carbonic anhydrase immobilized on standard bioreactor packing materials and a feed of bicarbonate solution from the absorber.

[00200] Additionally, these system designs can be combined in different configurations depending on the specific application or gas stream to be treated. For example, the system specifications can be tailored to the CO₂ content of the feed stream and the overall purity, recovery, and contaminant levels required for the product streams along with the temperature and pressure requirements of both streams. Moreover, a packed tower may be used as the absorber in conjunction with a membrane bioreactor as the desorber. Alternatively, a membrane bioreactor may be used as the absorber and a packed tower can be used as the desorber. Also, the system design may further include a carbon capture process unit that comprises a standard absorption unit and a stripping (reactive distillation) unit. The core components of the carbon capture system include, for example, an absorbing unit operation, a stripping unit operation, and a heat exchange component between the two unit operations. The above systems for accelerating the rate of

absorption and reaction of CO₂ are described in detail in WO 2010/037109.

[00201] Another aspect of the present disclosure relate to the use of any of the carbonic anhydrase polypeptide-containing compositions disclosed herein to regulate the pH of an aqueous solution. In certain embodiments, an effective amount of the composition is contacted with an aqueous solution under suitable conditions to regulate the pH of the solution to yield a solution having a desired pH value.

[00202] Other aspects and embodiments of the present compositions and methods will be apparent from the foregoing description and following examples.

EXAMPLES

[00203] The following examples are provided to demonstrate and illustrate certain preferred embodiments and aspects of the present disclosure and should not be construed as limiting.

[00204] In the experimental disclosure which follows, the following abbreviations apply: M (molar); mM (millimolar); μ M (micromolar); nM (nanomolar); mol (moles); mmol (millimoles); μ mol (micromoles); nmol (nanomoles); g and gm (grams); mg (milligrams); μ g (micrograms); ng (nanograms); pg (picograms); L (liters); ml and mL (milliliters); μ l and μ L (microliters); cm (centimeters); mm (millimeters); μ m (micrometers); nm (nanometers); U (units); MW (molecular weight); sec (seconds); min(s) (minute/minutes); h(s) and hr(s) (hour/hours); °C (degrees Centigrade); QS (quantity sufficient); ND (not done); rpm (revolutions per minute); H₂O (water); dH₂O (deionized water); HCl (hydrochloric acid); aa (amino acid); bp (base pair); kb (kilobase pair); kD (kilodaltons); Na₂SO₄ (sodium sulfate); MgCl₂ (magnesium chloride); NaCl (sodium chloride); Ca (calcium); Mg (magnesium); SO₄ (sulfate); SO_x (oxides of sulfur), NO_x (oxides of nitrogen), CO₂ (carbon dioxide); H₂SO₄ (sulfuric acid); NaHCO₃⁻ (sodium bicarbonate); KHCO₃⁻ (potassium bicarbonate); HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); CHES (N-cyclohexyl-2-aminoethanesulfonic acid); CAPS (N-cyclohexyl-3-aminopropanesulfonic acid); BSA (bovine serum albumin); w/v (weight to volume); v/v (volume to volume); ppm (parts per million); Bgi CA1 (*Bacillus gibsonii* carbonic anhydrase 1); Pvi CA1 (*Promicromonospora vindobonensis* carbonic anhydrase1); Ate CA1 (*Aspergillus terreus* carbonic anhydrase1); Spr CA1 (*Streptomyces pristinaespiralis* carbonic anhydrase1); Bag CA1 (*Bacillus agaradhaerens* carbonic anhydrase 1); Vsp CA1 (*Vibrio sp. AND4* carbonic anhydrase 1); and VspE CA1 (*Vibrio sp. EX25* carbonic anhydrase 1).

EXAMPLE 1

A. Cloning of *Bacillus gibsonii* carbonic anhydrase *Bgi CA1*

[00205] A carbonic anhydrase gene, *Bgi CA1*, was identified from the genome of *Bacillus gibsonii* DSM 8722 strain. The sequence of this gene is depicted in SEQ ID NO: 1. The sequence of the protein encoded by the *Bgi CA1* gene is depicted in SEQ ID NO: 2. The gene has an alternative start codon

(TTG). At the N-terminus, the protein has a signal peptide of 29 amino acids in length as predicted by SignalP-NN (Emanuelsson et al., Nature Protocols, 2:953-971, 2007). The presence of a signal sequence suggests that Bgi CA1 is a secreted enzyme.

[00206] The nucleotide sequence of the *Bgi CA1* gene isolated from *Bacillus gibsonii* is set forth as SEQ ID NO: 1:

TTGAAACGATCAAATCTACTTATTTAAAACGACAGTAGCCGCTACCATCTATTAACTAGTACATCCTTTT
TGACAGAAGCAATGCTTGACATGGAAATCACGTAAGTTCTCTCTTTAATTCATTACCCTATGATCG
TTTGACTGCGAACGCGTCCCATGATTGGTCATATTCTGGTCCAACAGGTCCTGAGTTTTGGGGAGAGCTT
GACTCTGAATTTAAAGCCTGCTCTAATGGCACGCAGCAATCCCCAATTGCACTAGACCCAACCGATGTTG
GCGATGAAAAATGGAGCTTGGACCTAGATTATGCCAAAACAGAGTTTTCCATTGAAAACAATGGTCATAC
CATTCAAGCCAATGTGGTTGAAAAAAAAGGACAGCCTTCCAATCAATTAACACTTGGCGACTCCACATAT
GAACTGGTTCAATTTTCAATTTTCACTACCGAGTGAGCATAACGCTAGCAGGAGAGTCTTATGAAATGGAAG
TACACCTTGTTCTATAAAGATGAGCAAGACAATCTTGCTGTGTTAGGCGTATTAATGGAAGAAGGAGAAAA
AAACAAAGCTTTAAAAGATATGTGGAAGAAGATGCCGACTAGTGTGCGAACTTCAACTAAAACCATTAAAG
TTAAATCCTAGTGAGCTGGTTCTACTGATCTATCAACTTTTCAATATGACGGTTCGCTTACTACCCCGC
CTTGCTCTGAAGGTGTGAAGTGGAGTGTGAGTGACTCTTCAATTACACTCTCTTCGGAACAGCTTCAAGC
TTTTCAAGATTTGTACCCGAATAACTATCGCCCAATTCAAGATTTAGGGGATCGTGAAGTTGGTTTTTCAT
TAT

[00207] The amino acid sequence of the Bgi CA1 precursor protein is set forth as SEQ ID NO: 2. the predicted native signal peptide is shown in italics.

*MKRSNLLIKTTVAATILLTSTSFLTEAM*LAHGNHVSSSSLIHSPYDRLTANASHDWSYSGPTGPEFWGEL
DSEFKACSNGTQQSPIALDPTDVGDEKWSLDLDYAKTEFSIENNGHTIQANVVEKKGQPSNQLTLGDSTY
ELVQFHFHSPSEHTLAGESYEMEVHLVHKDEQDNLAVLGLVLMEEGEKNKALKDMWKKMPTSVGTSTKTIK
LNPSSELVPTDLSTFQYDGSLTTPPCSEGVKWSVSDSSITLSSEQLQAFQDLYPNNYRPIQDLGDREVGFFH
Y

[00208] The amino acid sequence of the mature form of Bgi CA1 is set forth as SEQ ID NO: 3:

AHGNHVSSSSLIHSPYDRLTANASHDWSYSGPTGPEFWGELDSEFKACSNGTQQSPIALDPTDVGDEKWS
LDLDYAKTEFSIENNGHTIQANVVEKKGQPSNQLTLGDSTYELVQFHFHSPSEHTLAGESYEMEVHLVHK
DEQDNLAVLGLVLMEEGEKNKALKDMWKKMPTSVGTSTKTIKLNPSSELVPTDLSTFQYDGSLTTPPCSEGV
KWSVSDSSITLSSEQLQAFQDLYPNNYRPIQDLGDREVGFFHY

B. Expression of *Bacillus gibsonii* Carbonic Anhydrase (*Bgi CA1*)

[00209] The *Bgi CA1* gene was amplified from genomic DNA of *Bacillus gibsonii* by PCR. PCR was performed using a thermocycler with KOD-plus polymerase (TOYOKAI) according to the instructions of the manufacturer (annealing temperature of 59°C). The primers used were: BgiCA1-Fw 5'-GCGGCTAGCG CAGCACATGG AAATCACGTA AGTT-3' (SEQ ID NO: 4), and BgiCA1-Rv 5'-GCGGTAACT CATTATAAT GAAAACCAAC TTCACG-3' (SEQ ID NO: 5). In order to remove the

internal *NheI* site within the *Bgi CA1* gene, an overlap PCR was first carried out to introduce a point mutation in the *Bgi CA1* gene (A387 to G387). The primers used to insert the mutation were: BgiCA1-mu-Fw 5'-AGCATACGCT GGCAGGAGAG TCTTATGAAA TGG-3' (SEQ ID NO: 6), and BgiCA1-mu-Rv 5'-CTCTCCTGCC AGCGTATGCT CACTCGGTGA-3' (SEQ ID NO: 7).

5 [00210] The resulting PCR fragment of mature *Bgi CA1* DNA was digested with *NheI* and *HpaI* and ligated using T4 DNA ligase into pHPLT vector (*see*, U.S. Patent 6,566,112, containing the *B. licheniformis* LAT promoter (Plat), and additional elements from pUB110 (McKenzie et al., Plasmid, 15:93-103, 1986) including a replicase gene (reppUB), a neomycin/kanamycin resistance gene (neo) and a bleomycin resistance marker (bleo)) (50 ng/μL) digested with the same restriction enzymes to obtain

10 the expression plasmid pHPLT02-Bgi CA1 (Figure 1). The reaction conditions used for ligation were according to the instructions of the supplier (New England Biolabs, MA). The pHPLT02 vector contains the thermostable amylase LAT promoter (pLAT) and a signal peptide from *Bacillus licheniformis* strain DSM13 for expression of Bgi CA1. The vector can replicate in *B. subtilis*. The ligation mixture was amplified using a rolling circle kit (GE Healthcare Life Sciences, NJ). A multi-delete *B. subtilis* host

15 strain, which includes the deletion of a number of protease genes (including, e.g., the genes *nprE*, *aprE*, *nprB*, *vpr*, etc), was transformed with the amplified ligation mixture. The transformed cells were plated on Luria Agar plates supplemented with 10 ppm kanamycin. About 50-100 colonies were obtained, 24 of which were picked and grown in 24-well plates. The sequence of *Bgi CA1* gene was confirmed by DNA sequencing. Selected clones from the 24-well plates were further grown in a 7-L fermentor.

20 [00211] The nucleotide sequence of the *Bgi CA1* gene of plasmid pHPLT02-Bgi CA1 is set forth as SEQ ID NO: 8. The signal sequence is shown in *italics*, and the *NheI* restriction site is shown in **bold**.

ATGAAAAACATCCGCAAAACGGTCATCTTTGCGGCAATCATCCTGCTGGTCCATACAGCGGTTCCGGCAA
TCCCG**GCTAGC**GCAGCACATGGAAATCACGTAAGTTCTCTTCTTTAATTCATTACCCCTATGATCGTTT
GACTGCGAACGCGTCCCATGATTGGTCATATTCTGGTCCAACAGGTCCTGAGTTTTGGGGAGAGCTTGAC
25 TCTGAATTTAAAGCCTGCTCTAATGGCACGCAGCAATCCCCAATTGCACTAGACCCAACCGATGTTGGCG
ATGAAAAATGGAGCTTGGACCTAGATTATGCCAAAACAGAGTTTTCCATTGAAAACAATGGTCATACCAT
TCAAGCCAATGTGGTTGAAAAAAGGACAGCCTTCCAATCAATTAACACTTGGCGACTCCACATATGAA
CTGGTTCAATTTTCAATTTTCACTCACCGAGTGAGCATACTGCTGGCAGGAGAGTCTTATGAAATGGAAGTAC
ACCTTGTTTATAAAGATGAGCAAGACAATCTTGCTGTGTTAGGCGTATTAATGGAAGAAGGAGAAAAAA
30 CAAAGCTTTAAAGATATGTGGAAGAAGATGCCGACTAGTGTCGGAACCTCAACTAAAACCATTAAAGTTA
AATCCTAGTGAGCTGGTTCCTACTGATCTATCAACTTTTCAATATGACGGTTCGCTTACTACCCCGCCTT
GCTCTGAAGGTGTGAAGTGGAGTGTGAGTGACTCTTCAATTACACTCTCTTCGGAACAGCTTCAAGCTTT
TCAAGATTTGTACCCGAATAACTATCGCCCAATTCAAGATTTAGGGGATCGTGAAGTTGGTTTTTATTAT
TAA

35 [00212] The amino acid sequence of the Bgi CA1 precursor protein expressed from plasmid pHPLT02-Bgi CA1 is set forth as SEQ ID NO: 9:

MKNIRKTVIFAAIILLVHTAVPAIPASAAHGNHVSSSLI HSPYDRLTANASHDWSYSGP
 TGPEFWGELDSEFKACSNGTQQSPIALDPTDVGDEKWSLDLDYAKTEFSIENNGHTIQANVVEKKQPSN
 QLTLDSTYELVQFHFHSPSEHTLAGESYEMEVHLVHKDEQDNLAVLGVLMEEGEKNKALKDMWKKMPTS
 VGTSTKTIKLNPSSELVPTDLSTFQYDGSLLTPPCSEGVKWSVSDSSITLSSEQLQAFQDLYPNNYRPIQD
 5 LGDREVGPHY

[00213] Several signal sequences were used to express Bgi CA1. These sequences are listed in Table 1B-1.

Table 1B-1: Signal sequences used to express Bgi CA1

SEQ ID	Signal Sequence (SS)
NO:10	MKNIRKTVIFAAIILLVHTAVPAIPASA
NO:11	MIKKWAVHLLFSALVLLGLSGASA
NO:12	MAAEKVFSKNKIIGGKRMSYMKRSISVFIACFMVAALGISGIIAPKASA
NO:13	MKKTIMSLAAAAAMSATAFGATASA
NO:14	MKKFACVVIFLLLA AVIAGCAADASA
NO:15	MKKRLMSLLVCILVLVPAAGASA

Protein purification of Bgi CA1

[00214] Bgi CA1 protein was purified from concentrated broth from a 7-L fermentor run using three chromatography columns: 1) a phenyl sepharose column equilibrated with 20 mM Tris HCl buffer, pH 8.0, containing 1 M ammonium sulfate, from which the protein was eluted in the void volume; 2) an anion exchange Q sepharose column equilibrated with 20 mM Tris HCl buffer, pH 8.0, from which the protein was eluted using a linear gradient of equilibration/wash buffer to 20 mM Tris HCl, pH 8.0 buffer containing 0.5 M NaCl; 3) a Superdex 75 gel filtration column, from which the protein was eluted using 20 mM sodium phosphate, pH 7.0, containing 0.15 M NaCl. The purified protein fractions were pooled and concentrated using a 3K Amicon Ultra-15 device and the concentrated protein fraction was used in further studies.

C. Carbonic Anhydrase Activity of Bgi CA1

[00215] The carbonic anhydrase activity of purified Bgi CA1 was measured on ice in 20 mM Tris sulfate buffer (pH 8.3 at 25°C) containing 20 mM Na₂SO₄. Briefly, 3 mL of chilled 20 mM Tris sulfate buffer (pH 8.3 at 25°C) containing 20 mM Na₂SO₄ was added to a 50-mL polypropylene conical tube placed on ice. 20 µL of enzyme sample or buffer was added to the tubes followed by 2 mL of chilled CO₂ saturated water (purified and deionized using Milli-Q Integral system, Millipore). After the contents were mixed, a standardized pH meter probe was immediately inserted into the solution and the time required for the pH to drop from 8.3 to 6.3 was recorded using a stop watch. The test was repeated until at least three blank measurements (with buffer added) were within a 15-second time window and the average of three determinations ($T_{\text{Blank,avg}}$) was between 70 and 100 seconds. A standard curve was

prepared using bovine carbonic anhydrase II (bCAII, Sigma C2522). The test measurements for determining activity were performed in triplicate, and $T_{\text{enzyme,avg}}$ was obtained.

[00216] One unit of activity (Wilbur-Anderson (W-A) unit, Wilbur and Anderson, *J. Biol. Chem.* 176, 147-154, 1948) is defined as the time required for the pH of a 0.02 M Trizma buffer to drop from 8.3 to 6.3 per min at 0°C:

$$\text{Units/mL enzyme} = (T_{\text{Blank,avg}} - T_{\text{enzyme,avg}}) * \text{DF} / (T_{\text{enzyme,avg}} * V)$$

where DF = dilution factor of enzyme sample, V = volume (in mL) of enzyme used, $T_{\text{Blank,avg}}$ = average time values of Blank measurements, and $T_{\text{enzyme,avg}}$ = average time values of Test measurements.

$$\text{Units/mg protein} = (\text{units/mL enzyme}) / (\text{mg protein/mL enzyme})$$

[00217] The specific activity of purified Bgi CA1 was determined to be 4023 ± 387 units/mg using the above method. Carbonic anhydrase activity of Bgi CA1 is suitable for enzyme based CO₂ extraction.

D. Temperature Stability of Bgi CA1

[00218] The temperature stability of Bgi CA1 was determined in 0.1 M Tris buffer, pH 8.5 (adjusted by H₂SO₄ at 25°C), 0.1 M CHES buffer, pH 9.5 (adjusted by H₂SO₄ at 25°C), and 0.1 M CAPS buffer, pH 10.5 (adjusted by H₂SO₄ at 25°C). Na₂SO₄ was added to the buffer to a final concentration of 25 mM. 100 ppm Bgi CA1 in buffer, in 20 mL final volume, was incubated at room temperature (25°C), or in a water bath at 40°C and 50°C. At different time points (5 minutes to 24 hours), 0.5 mL of the enzyme sample from each tube was taken and placed on ice. The specific activity of each enzyme sample was measured as described in Example 1C. The specific activity measurements were performed in triplicates. The percent remaining activity was calculated at each time point and at each pH value. For each pH value, the activity of the sample at time 0 was defined as 100% activity. As shown in Figures 2A-C, Bgi CA1 retained more than 90% activity over a 24-hour incubation period at 25°C and 40°C. At 50°C, at least 70% of the activity was retained after a 3-hour incubation period at pH 8.5 and pH 9.5, and 25% activity was retained at pH 10.5. Thus, Bgi CA1 is stable over different pH ranges typically used for enzymatic CO₂ extraction, and at supra-physiological temperatures.

E. Stability of Bgi CA1 in 1M NaHCO₃

[00219] The stability of Bgi CA1 under high ionic strength condition was determined in a 1 M NaHCO₃ solution. In a PCR machine, a 100 ppm solution of Bgi CA1 in 1 M NaHCO₃ was incubated at 20°C and 50°C. The specific activities of the samples were measured as described in Example 1C. Prior to incubation and at varying time points (5 minutes to 3 hours), 100 µL samples were withdrawn, cooled on ice and their specific activity measured. The specific activity measurements were performed in

triplicates. The percent remaining activity was calculated for each time point. The activity of the sample kept on ice at time 0 was defined as 100% activity. Figure 3 shows stability of Bgi CA1 in 1 M NaHCO₃. Bgi CA1 retains most of the activity over a 3-hour incubation period at both 20°C and 50°C. Thus, Bgi CA1 is stable in the presence of high concentrations of NaHCO₃ that are encountered in enzymatic CO₂ extraction.

F. Heat Capacity Measurement of Bgi CA1

[00220] Excessive heat capacity curves were measured for Bgi CA1 and bovine carbonic anhydrase II (bCA II) using an ultrasensitive scanning high-throughput microcalorimeter, VP-Cap DSC (MicroCal, Inc., Northampton, MA). The standard procedure for DSC measurements and the theory of the technique has been described previously (Freire, Differential Scanning Calorimetry, *Methods Mol. Biol.*, 41:191-218, 1995). About 500 µL of a 0.5 mg/mL sample of each enzyme was studied. The proteins were scanned over a 35-100°C temperature range. The same samples were then re-scanned (following cooling) to check the reversibility of the thermal unfolding process. For both carbonic anhydrases studied the thermal unfolding was irreversible. The proteins were studied in the following buffers: (1) 0.1 M Tris, pH 8.5, (2) 0.1 M CHES, pH 9.5, (3) 0.1 M CAPS, pH 10.5, and (4) 0.1 M CHES, pH 9.5 + 2 M NaCl. A 200°C/hr scan rate was used to minimize any artifacts that might have resulted from aggregation. The apparent thermal midpoint [$T_m(\text{app})$] of the DSC curves was used as an indicator of the thermal stability and melting points for these carbonic anhydrase molecules. The melting temperature ($T_m(\text{app})$) of each enzyme in various buffers is shown in Table 1F-1.

[00221] The $T_m(\text{app})$ values during thermal unfolding for both the carbonic anhydrase proteins showed a dependence on pH over the range 8.5-to-10.5. Bovine carbonic anhydrase II has highest $T_m(\text{app})$ values between pH 8.5 and 9.5, while Bgi CA1 has the highest $T_m(\text{app})$ values at pH 8.5. The buffer of 0.1 M CHES, pH 9.5, with 2 M NaCl decreased the $T_m(\text{app})$ for bovine CA II, and increased the $T_m(\text{app})$ for Bgi CA1. For Bgi CA1, the carbonate buffer increased the $T_m(\text{app})$ substantially, and the 0.1 M CHES, pH 9.5 with 2 M NaCl increased the T_m by about 15°C. Thus, Bgi CA1 is suitable for enzymatic CO₂ extraction at higher temperatures because the T_m of this enzyme increases in carbonate solution and under high ionic strength conditions typical of enzymatic CO₂ extraction.

Table 1F-1: Melting Temperature for Bgi CA1 and bCAII

	Buffers			
	#1	#2	#3	#4
Protein	0.1 M Tris, pH 8.5	0.1 M CHES, pH 9.5	0.1 M CAPS, pH 10.5	0.1 M CHES, pH 9.5 + 2 M NaCl
Bovine CA II	68.8	69.4	66.6	61.7
Bgi CA1	59.9	56.9	56.6	71.9

[00222] $T_m(\text{app})$ was also determined for bCAII and Bgi CA1 in 1 M KHCO₃ with no added NaCl and at various concentrations of added NaCl, ranging from 0.1 M to 1 M (Table 1F-2). $T_m(\text{app})$

decreased for bCAII in bicarbonate solution as compared to $T_m(\text{app})$ in buffer, while $T_m(\text{app})$ increased for Bgi CA1 in bicarbonate solution, and with increased ionic strength. This increase in $T_m(\text{app})$ indicated the suitability of the enzyme Bgi CA1 in enzymatic CO_2 extractions, especially in the presence of high concentrations of carbonate solutions and at high ionic strength conditions. It was also noted that the $T_m(\text{app})$ of Bgi CA1 increases with increasing KHCO_3 concentration, which was unexpected.

Table 1F-2: $T_m(\text{app})$ [$^{\circ}\text{C}$] of Bgi CA1 in 1 M KHCO_3

[Salt] (M)	bCA II	Bgi CA1
0	62.1	70.9
0.1	62.8	71.4
0.5	62.5	74.1
1	62.7	76.6

G. Homology Identification

[00223] The Bgi CA1 mature protein sequence (252 residues) was subjected to a BLASTP search, and the top ten protein sequences were selected for sequence alignment (Vector NTI, Invitrogen). Table 1G-1 shows the percent identities and NCBI Accession Nos. of the aligned sequences NP_241226 (SEQ ID NO:16), NP_767777 (SEQ ID NO:17), NP_946147 (SEQ ID NO:18), YP_003428428 (SEQ ID NO:19), YP_003868697 (SEQ ID NO:20), YP_003944556 (SEQ ID NO:21), YP_176677 (SEQ ID NO:22), YP_488219 (SEQ ID NO:23), ZP_04209878 (SEQ ID NO:24), and ZP_04248491 (SEQ ID NO:25). The aligned sequences were used to prepare a phylogenetic tree using the program MEGA 5 (*see*, Tamura K. et al. Molecular Biology and Evolution, 2011, in press). Figure 5 shows the phylogenetic tree for Bgi CA1 and its homologs.

Table 1G-1: Percent Identity Shared by Bgi CA1 and Similar Sequences

Homolog	SEQ ID NO:	% Identity (PID)
NP_241226	16	42
NP_767777	17	40
NP_946147	18	43
YP_003428428	19	43
YP_003868697	20	40
YP_003944556	21	40
YP_176677	22	51
YP_488219	23	40
ZP_04209878	24	42
ZP_04248491	25	38

EXAMPLE 2

A. Cloning of *Promicromonospora vindobonensis* carbonic anhydrase1 *Pvi CAI*

[00224] A carbonic anhydrase gene, *Pvi CAI*, was identified from the genome of

5 *Promicromonospora vindobonensis* YIM65009 strain. The sequence of this gene is depicted in SEQ ID NO: 26. The protein encoded by the *Pvi CAI* gene is depicted in SEQ ID NO: 27. The gene has an alternative start codon (GTG). At the N-terminus, the protein has a signal peptide with a length of 25 amino acids as predicted by SignalP-NN (Emanuelsson *et al.*, *Nature Protocols*, 2: 953-971, 2007). The presence of a signal sequence suggests that Pvi CA1 is a secreted enzyme.

10 [00225] The nucleotide sequence of the *Pvi CAI* gene isolated from *Promicromonospora vindobonensis* is set forth as SEQ ID NO: 26:

GTGAAGAAGCTCGCCCTGCCCACCGTCCTGCTCCTCGCCCCGTTGCTCGCGTCCTGCGCGTCGGGCACCG
CCGCCGACGGCGAGACGTCCGCACCCCCGCCGCCGCGACCGACGAGGTGCACTGGTCCTACGAAGGGGA
CACGGGCCCGGACAACCTGGGGCCAGCTCTCCGACGAGTTTCGTGAGTGCTCGATCGGCGAGGCGCAGTCC
15 CCGGTGACCTGCCGGACACGCCGACGAGACGACCACCGAGCCCCCGACGGTCACGACGTGGCCACCG
TCGGCGAGTCGGTCGACACCGGCCACACGATCCAGCTCGTGCCCGACGGCGACGCGTCCGAGGTGAGTG
GCAGGACACCACGTTTCGACCTCGCCAGGTGCACTTCCACATGCCCTCGGAGCACACGATCGAGGGTGAG
GCGCTCGACGCCGAGTTCCACTTCGTCCACACCACGGAGGAAGGACAGGCGCTCGTCATCGGGGTCTCTCG
CGCGGGAGAGCAGCACCGAGAACGAGGCCTGGCAGCCGTTTCATCGATGGTGCGGCCGAGCCGGGCACCGA
20 GGACCTGCCGCTCGACGTGCGCCGCGATGCTACCGACGGACCCGACGTTTCGAGGAGTACACGGGCAGCCTC
ACGACCCCGCCGTGCACCGAGGGCGTCGAGTGGGTCTGTCTACACGAGCCCATCGAGCTGTTCGGCGGAGC
AGATCGCCGTGCTCAGGGACGCGTACGACAACACCGCGCGCCCGACCCAGCTCCTGGGCGACCGCGTCGT
GTACGAGGGCACCATCGACGTGGAGGCGGAGGAGGCGCAC

[00226] The amino acid sequence of the Pvi CA1 precursor protein is set forth as SEQ ID NO: 27.

25 The predicted signal peptide is shown in italics.

MKKLALPTVLLLAPLLASCASGTAADGETSAPPPPATDEVHWSYEGDTGPDNWGQLSDEFVECSIGEAQS
PVDLPDHADETTTEPPTVTWPTVGESVDTGHTIQLVPDGDASEVEWQDITFDLAQVHFHMPSEHTIEGE
ALDAEFHFVHTTEEGQALVIGVLARESSTENEAWQPFIDGAAEPGTEDLPLDVAAMLPTDPTFEEYTGSL
TTPPCTEGVEWVYHEPIELSAEQIAVLRDAYDNTARPTQLLGDRVVYEGTIDVEAEEAH

30 [00227] The amino acid sequence of the mature form of Pvi CA1 is set forth as SEQ ID NO: 28:

DGETSAPPPPATDEVHWSYEGDTGPDNWGQLSDEFVECSIGEAQSPVDLPDHADETTTEPPTVTW
WPTVGESVDTGHTIQLVPDGDASEVEWQDITFDLAQVHFHMPSEHTIEGEALDAEFHFVHTTEE
GQALVIGVLARESSTENEAWQPFIDGAAEPGTEDLPLDVAAMLPTDPTFEEYTGSLTTPPCTEGV
EWVVYHEPIELSAEQIAVLRDAYDNTARPTQLLGDRVVYEGTIDVEAEEAH

B. Expression of *Promicromonospora vindobonensis* Carbonic Anhydrase1 (*Pvi CAI*)

[00228] The *Pvi CAI* gene was amplified from genomic DNA of *Promicromonospora vindobonensis* by PCR. PCR was performed on a thermocycler with KOD-plus polymerase (TOYOBA) according to the instructions of the manufacturer (annealing temperature of 59°C). The primers used were: PviCA1-Fw
 5 5'-AGCGCTAGCC GGCCCCCGG CACAGGCCGA CGGCGAGACG TCCGCACCCC-3' (SEQ ID NO: 29),
 and PviCA1-RV 5'-TCCGGATCCT TAGTGCGCCT CCTCCGCCTCCACGT-3' (SEQ ID NO: 30).

[00229] The resulting PCR fragment of mature *Pvi CAI* DNA was digested with *NheI* and *BamHI*, and ligated into the pKB128 vector, and then digested with the same restriction enzymes to obtain the expression plasmid pJG183 (Figure 6). The pKB128 plasmid is a derivative of the pKB105 plasmid (as described in U.S. Patent Application Publication No. 2006/0154843) and is the source of the A4 promoter-CelA signal sequence. The ligation mixture was used to transform *E. coli* TOP10 chemically competent cells (Invitrogen Corp.) following the manufacturer's protocol. The transformed cells were plated on Luria Agar plates supplemented with 50 ppm ampicillin and incubated overnight at 37°C. Three transformants were picked from the plate and inoculated into 5 mL Luria Broth supplemented with
 15 50 ppm ampicillin. Cultures were grown overnight at 37°C, plasmid DNA extracted and the correct sequence of the *Pvi CAI* gene was confirmed by DNA sequencing. The pJG183 plasmid was then used to transform *S. lividans* TK23 derived protoplasts (as described in U.S. Patent Application Publication No. 2006/0154843). The transformation techniques used are described in Kieser et al., *Practical Streptomyces Genetics*, The John Innes Foundation, Norwich, United Kingdom, 2000. Transformed cells
 20 were plated on R5 selection plates and incubated at 30°C for 3 days. One clone from the *Streptomyces* transformation plate was inoculated in TSG medium in shake flasks at 28°C for 3 days. Cultures were then transferred to a *Streptomyces* 2 Modified Medium (as described in U.S. Patent Application Publication No. 2006/0154843) and incubated for an additional 4 days at 28°C.

[00230] The TSG medium was prepared using the following ingredients: 16 g BD Difco tryptone, 4 g
 25 BD Bacto soytone, 20 g Sigma caseine (hydrolysate), and 10 g potassium phosphate, dibasic, brought to 1 L. After autoclaving, 50% glucose was added to a final concentration of 1.5%.

[00231] The R5 plates were prepared using the following ingredients: 206 g sucrose, 0.5 g K₂SO₄, 20.24 g MgCl₂, 20 g glucose, 0.2 g Difco casamino acids, 10 g Difco yeast extracts, 11.46 g TES, 4 g L-Asp, 4 mL of trace elements, 44 g Difco agar, 20 mL 5% K₂HPO₄, 8 mL 5 M CaCl₂•2H₂O and 14 mL 1
 30 N NaOH were added to a final volume of 1 L after autoclaving. After 20 hours, a layer of thiostrepton (50 µg/mL final concentration) was plated on the top of the plates.

[00232] The nucleotide sequence of the *Pvi CAI* gene of plasmid pJG183 (A4-celA-PviCA1) is set forth as SEQ ID NO: 31:

GACGGCGAGACGTCCGCACCCCCGCCGCCGACCGACGAGGTGCACTGGTCCTACGAAGGGGACACGG
 35 GCCCCGACAACCTGGGGCCAGCTCTCCGACGAGTTCGTGAGTGCTCGATCGGCGAGGCGCAGTCCCCCGT

CGACCTGCCGGACCACGCCGACGAGACGACCACCGAGCCCCGACGGTCACGACGTGGCCCACCGTCGGC
 GAGTCGGTCGACACCGGCCACACGATCCAGCTCGTGCCCGACGGCGACGCGTCCGAGGTGAGTGCCAGG
 ACACCACGTTTCGACCTCGCCCAGGTGCACTTCCACATGCCCTCGGAGCACACGATCGAGGGTGAGGCGCT
 CGACGCCGAGTTCCACTTCGTCCACACCACGGAGGAAGGACAGGCGCTCGTCATCGGGGTCTCTCGCGCGG
 5 GAGAGCAGCACCGAGAACGAGGCCTGGCAGCCGTTTCATCGATGGTGCGGCCGAGCCGGGCACCGAGGACC
 TGCCGCTCGACGTCGCCGCGATGCTACCGACGGACCCGACGTTTCGAGGAGTACACGGGCAGCCTCACGAC
 CCCGCCGTGCACCGAGGGCGTCGAGTGGGTCGTCTACCACGAGCCCATCGAGCTGTCGGCGGAGCAGATC
 GCGGTGCTCAGGGACGCGTACGACAACACCGCGCGCCCCGACCCAGCTCCTGGGCGACCGCGTCGTGTACG
 AGGGCACCATCGACGTGGAGGCGGAGGAGGCGCAC

10 *Protein purification of Pvi CA1*

[00233] *Streptomyces lividans* cells expressing Pvi CA1 were grown in shake flasks under standard conditions. The Pvi CA1 was purified from fermentation broth using two chromatography columns: 1) an anion exchange Q sepharose column equilibrated with 20 mM Tris HCl buffer, pH 8.0, from which the protein was eluted using a linear gradient of equilibration/wash buffer to 20 mM Tris HCl, pH 8.0 buffer
 15 containing 0.5 M NaCl; 2) a Superdex 75 gel filtration column, from which the protein was eluted using 20 mM sodium phosphate, pH 7.0, containing 0.15 M NaCl. The purified protein fractions were pooled and concentrated using a 3K Amicon Ultra-15 device and the concentrated protein fraction was used in further studies.

C. Carbonic Anhydrase Activity of Pvi CA1

20 [00234] The carbonic anhydrase activity of purified Pvi CA1 was measured on ice in 20 mM Tris sulfate buffer (pH 8.3 at 25°C) containing 20 mM Na₂SO₄. Briefly, 3 mL of chilled 20 mM Tris sulfate buffer (pH 8.3 at 25°C) containing 20 mM Na₂SO₄ was added to a 50-mL polypropylene conical tube placed on ice. 20 µL of enzyme sample or buffer was added to the tubes followed by 2 mL of chilled CO₂ saturated water (purified and deionized using Milli-Q Integral system, Millipore). After the contents
 25 were mixed, a standardized pH meter probe was immediately inserted into the solution and the time required for the pH to drop from 8.3 to 6.3 was recorded using a stop watch. The test was repeated until at least three blank measurements (with buffer added) were within a 15-second time window and the average of three determinations ($T_{\text{Blank,avg}}$) was between 70 and 100 seconds. A standard curve was prepared using bovine carbonic anhydrase II (bCAII, Sigma C2522). The test measurements for
 30 determining activity were performed in triplicate, and $T_{\text{enzyme,avg}}$ was obtained.

[00235] One unit of activity (Wilbur-Anderson or W-A unit, Wilbur and Anderson, *Journal of Biological Chemistry* 176, 147-154, 1948) is defined as the time required for the pH of a 0.02 M Trizma buffer to drop from 8.3 to 6.3 per min at 0°C:

$$\text{Units/mL enzyme} = (T_{\text{Blank,avg}} - T_{\text{enzyme,avg}}) * \text{DF} / (T_{\text{enzyme,avg}} * V)$$

5 where DF = dilution factor of enzyme sample, V = volume (in mL) of enzyme used, $T_{\text{Blank,avg}}$ = average time values of Blank measurements, and $T_{\text{enzyme,avg}}$ = average time values of Test measurements.

$$\text{Units/mg protein} = (\text{units/mL enzyme}) / (\text{mg protein/mL enzyme})$$

10

[00236] The specific activity of purified Pvi CA1 was determined to be 937 ± 65 units/mg using the above method. Carbonic anhydrase activity of Pvi CA1 is suitable for enzyme based CO₂ extraction.

D. Temperature Stability of Pvi CA1

[00237] The temperature stability of Pvi CA1 was determined in 0.1 M Tris buffer, pH 8.5 (adjusted by H₂SO₄ at 25°C), 0.1 M CHES buffer, pH 9.5 (adjusted by H₂SO₄ at 25°C), and 0.1 M CAPS buffer, pH 10.5 (adjusted by H₂SO₄ at 25°C). Na₂SO₄ was added to the buffer to a final concentration of 25 mM. 100 ppm of Pvi CA1 in buffer, in 20 mL final volume, was incubated in a water bath at 40°C and 50°C. One sample was also incubated at the room temperature (25°C). At different time points (5 minutes to 24 hours), 0.5 mL of the enzyme sample from each tube was taken and placed on ice. The specific activity of each enzyme sample was measured as described in Example 2C. The specificity activity measurements were performed in triplicates. The percent remaining activity was calculated at each time point, at each pH value. For each pH value, the activity of the sample kept at 0°C at time 0 was defined as 100% activity. The Pvi CA1 retained 80% activity at 25°C at pH 8.5 and pH 9.5. At 40°C, the enzyme retained more than 70% activity at pH 8.5 and pH 9.5 for 27 hrs, and 30% activity at pH 10.5 for 3 hrs. Stability of Pvi CA1 is thus suitable for enzymatic CO₂ sequestration processes at different pH values. As shown in Figures 7A-C, Pvi CA1 is stable over different pH ranges typically used for enzymatic CO₂ extraction, and at supra-physiological temperatures.

E. Stability of Pvi CA1 in 1 M NaHCO₃

[00238] The stability of Pvi CA1 under high ionic strength conditions was assessed. In a PCR machine, a 100 ppm solution of Pvi CA1 in 1 M NaHCO₃ was incubated at 20°C and 50°C. The specific activities of the samples were measured prior to incubation as described in Example 2C. At varying time points (5 minutes to 3 hours), 100 µL samples were withdrawn, cooled on ice and their specific activity measured as described in Example 2C. The percent remaining activity was calculated for each time point. The activity of the sample kept on ice at time 0 was defined as 100% activity. Figure 8 shows stability of Pvi CA1 in 1 M NaHCO₃. Pvi CA1 retains most of the activity over a 3-hr incubation period

at both 20°C and 50°C. Thus, Pvi CA1 is stable in the presence of high concentrations of NaHCO₃ that are encountered in enzymatic CO₂ extraction.

F. Heat Capacity Measurement of Pvi CA1

[00239] Excessive heat capacity curves were measured for Pvi CA1 and bovine carbonic anhydrase II (bCA II) using an ultrasensitive scanning high-throughput microcalorimeter, VP-Cap DSC (MicroCal, Inc., Northampton, MA). The standard procedure for differential scanning calorimetry (DSC) measurements and the theory of the technique has been described previously (Freire, *Methods Mol Biol*, 41:191-218, 1995). About 500 µL of a 0.5 mg/mL sample of each enzyme was studied. The proteins were scanned over a 35-100°C temperature range. The same samples were then re-scanned (following cooling) to check the reversibility of the thermal unfolding process. For both carbonic anhydrases studied the thermal unfolding was irreversible. The proteins were studied in the following buffers: (1) 0.1 M Tris, pH 8.5, (2) 0.1 M CHES, pH 9.5, (3) 0.1 M CAPS, pH 10.5, and (4) 0.1 M CHES, pH 9.5 + 2 M NaCl. A 200°C/hr scan rate was used to minimize any artifacts that might have resulted from aggregation. The apparent thermal midpoint [$T_m(\text{app})$] of the DSC curves was used as an indicator of the thermal stability and melting temperature for these carbonic anhydrase molecules. The melting temperature ($T_m(\text{app})$) of each enzyme in various buffers is shown in Table 2F-1.

[00240] The $T_m(\text{app})$ values during thermal unfolding for both the carbonic anhydrase proteins showed a dependence on pH over the range of pH 8.5 to pH 10.5. Bovine carbonic anhydrase II has highest $T_m(\text{app})$ values between pH 8.5 and pH 9.5, while Pvi CA1 has the highest $T_m(\text{app})$ values at pH 8.5. The buffer of 0.1 M CHES, pH 9.5, with 2 M NaCl decreased the $T_m(\text{app})$ for bovine CA II, and increased the $T_m(\text{app})$ for Pvi CA1. For Pvi CA1, the buffer of 0.1 M CHES, pH 9.5 with 2 M NaCl increased the $T_m(\text{app})$ by about 6°C compared to 0.1 M CHES buffer, pH 9.5 with no additives. Thus, Pvi CA1 is suitable for enzymatic CO₂ extraction at higher temperatures because the T_m of this enzyme increases in carbonate solution and under high ionic strength conditions typical of enzymatic CO₂ extraction.

Table 2F-1: Melting Point Temperature for Pvi CA1 and bCAII

	Buffers			
	#1	#2	#3	#4
Protein	0.1 M Tris, pH 8.5	0.1 M CHES, pH 9.5	0.1 M CAPS, pH 10.5	0.1 M CHES, pH 9.5 + 2 M NaCl
Bovine CA II	68.8	69.4	66.6	61.7
Pvi CA1	70.5	66.9	62.6	73.7

[00241] $T_m(\text{app})$ was also determined for bCAII and Pvi CA1 in 1 M KHCO₃ with no added NaCl, at various concentrations of added NaCl, ranging from 0.1 M to 1 M (Table 2F-2). $T_m(\text{app})$ decreased for bCAII in bicarbonate solution as compared to $T_m(\text{app})$ in buffer, while $T_m(\text{app})$ increased for Pvi CA1 in bicarbonate solution, and with increased ionic strength. This increase in $T_m(\text{app})$ indicated the suitability

of the enzyme Pvi CA1 for enzymatic CO₂ extraction, especially in the presence of high concentrations of carbonate solutions and at high ionic strength conditions. This observation of a higher T_m(app) at higher ionic strength was unexpected.

Table 2F-2: T_m(app) [°C] of Pvi CA1 in 1 M KHCO₃

[Salt] (M)	bCA II	Pvi CA1
0	62.1	72.1
0.1	62.8	73.3
0.5	62.5	74.6
1	62.7	75.8

5

G. Homology Identification

[00242] The Pvi CA1 mature protein sequence (245 residues) was subjected to a BLASTP search, and the top ten protein sequences were selected for sequence alignment (Vector NTI, Invitrogen). Figure 9 shows an alignment of Pvi CA1 with similar carbonic anhydrase sequences. Table 2G-1 shows the percent identities and NCBI Accession Nos. of the aligned sequences: NP_241226 (SEQ ID NO: 16), YP_003166850 (SEQ ID NO: 32), YP_003610599 (SEQ ID NO: 33), YP_003868697 (SEQ ID NO: 20), YP_003944556 (SEQ ID NO: 21), YP_287120 (SEQ ID NO: 34), YP_419944 (SEQ ID NO: 35), ZP_00056365 (SEQ ID NO: 36), ZP_05029585 (SEQ ID NO: 37), and ZP_08017465 (SEQ ID NO: 38). The aligned sequences were used to prepare a phylogenetic tree using the program MEGA 5 (*see*, Tamura K. et al. Molecular Biology and Evolution, 2011, in press)). Figure 10 shows the phylogenetic tree for Pvi CA1 and its homologs.

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Table 2G-1: Percent Identity Shared by Pvi CA1 and Similar Sequences

Homolog	SEQ ID NO:	% IDENTITY (PID)
NP_241226	16	39
YP_003166850	32	38
YP_003610599	33	37
YP_003868697	20	36
YP_003944556	21	36
YP_287120	34	34
YP_419944	35	39
ZP_00056365	36	39
ZP_05029585	37	37
ZP_08017465	38	38

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EXAMPLE 3

A. Sequence of *Aspergillus terreus* Carbonic Anhydrase1 (Ate CA1)

[00243] Genomic DNA of *Aspergillus terreus* NIH2624 was purchased from Fungal Genetics Stock Center, Kansas City, MO (FGSC A1156). The nucleic acid sequence for the *Ate CA1* gene (NCBI Reference Sequence NT_165972.1), and the amino acid sequence of the hypothetical protein encoded by the *Ate CA1* gene was found in the NCBI Databases (NCBI Accession No. XP_001210252).

B. Expression of *Aspergillus terreus* Carbonic Anhydrase1 (Ate CA1)

[00244] The Ate CA1 gene was amplified from the genomic DNA of *Aspergillus terreus* NIH2624 by PCR. PCR was performed on a thermocycler with KOD-plus polymerase (TOYOBA) according to the instructions of the manufacturer, with an annealing temperature of 59°C). Primer design was based on the sequence of the *Ate CA1* genomic DNA. The following primers were used to amplify the *Ate CA1* gene: AteCA1-Fw 5'-GCGGCGGCCG CACCATGAAG CTCACTGCTG CCGTT-3' (SEQ ID NO: 39); and AteCA1-Rv 5'-CCGGCGCGCC CTTACTAGTT GAGGCTCTTG GCCG-3' (SEQ ID NO: 40). The forward primer contains a *NotI* restriction site, and the reverse primer contains an *AscI* restriction site.

[00245] The PCR product was purified, digested with *NotI* and *AscI*, ligated into plasmid vector pTTT (derived from vector pTTT, as described in US2011/0020899A1), and digested with the same restriction enzymes to obtain pTTT-Ate CA1. The plasmid map of pTTT-Ate CA1 is shown in Figure 11. *E. coli* TOP10 chemically competent cells (Invitrogen Corp.) were transformed with the ligation mixture following the manufacturer's protocol. The transformed cells were plated on Luria Agar plates supplemented with 50 ppm ampicillin and incubated overnight at 37°C. Three transformants from the plates were picked, inoculated in 5 mL of Luria Broth supplemented with 50 ppm ampicillin, and grown overnight. Plasmid DNA extracted from these cultures was sent for sequence confirmation.

[00246] Following sequence confirmation, protoplasts of a quad deleted *T. reesei* strain (as described in WO 05/001036) were transformed with the expression plasmid pTTT-Ate CA1 using the PEG protoplast method (Penttila et al, *Gene*, 61:155-164, 1987). For protoplast preparation, spores were grown for about 10 hours at 24°C in *Trichoderma* Minimal Medium MM (20 g/L glucose, 15 g/L KH₂PO₄, pH 4.5, 5 g/L (NH₄)₂SO₄, 0.6 g/L MgSO₄·7H₂O, 0.6 g/L CaCl₂·2H₂O, 1 mL of 1000X *T. reesei* Trace elements solution (175 g/L Citric Acid anhydrous, 200 g/L FeSO₄·7H₂O, 16 g/L ZnSO₄·7H₂O, 3.2 g/L CuSO₄, 1.4 g/L MnSO₄·H₂O, and 0.8 g/L Boric Acid). Germinating spores were harvested by centrifugation and treated with 30 mg/mL Vinoflow FCE (Novozymes, AG Switzerland) solution for from 7 hours to overnight at 30°C at 100 rpm to lyse the fungal cell walls. Protoplasts were washed in 0.1 M Tris HCl buffer (pH 7) containing 0.6 M sorbitol and resuspended in 10 mM Tris HCl buffer (pH 7.5) containing 1.2 M sorbitol and 10 mM calcium chloride. For PEG transformation, approximately 1 µg of DNA and 1-5 x 10⁷ protoplasts in a total volume of 200 µL were treated with 2 mL of 25% PEG solution, diluted with 2 volumes of 1.2 M sorbitol/10 mM Tris, pH 7.5/10 mM CaCl₂

solution. Transformants were selected on a medium containing acetamide as a sole source of nitrogen (acetamide 0.6 g/L; cesium chloride 1.68 g/L; glucose 20 g/L; potassium dihydrogen phosphate 15 g/L; magnesium sulfate heptahydrate 0.6 g/L; calcium chloride dihydrate 0.6 g/L; iron (II) sulfate 5 mg/L; zinc sulfate 1.4 mg/L; cobalt (II) chloride 1 mg/L; manganese (II) sulfate 1.6 mg/L; agar 20 g/L; pH 4.25). Transformed colonies (about 50-100) appeared in about 1 week. After growth on acetamide plates, the spores were collected and reselected on acetamide plates. After 5 days, the spores were collected using 10% glycerol, and 1×10^8 spores were inoculated in a 250 mL shake flask with 30 ml Glucose/Sophorose defined medium for protein expression. Protein expression was confirmed by SDS-PAGE. The spore suspension was subsequently grown in a 7 L fermentor in a defined medium containing 60% glucose-sophorose feed. Glucose/Sophorose defined medium (per liter) consists of (NH₄)₂SO₄ 5 g, PIPPS buffer 33 g, Casamino Acids 9 g, KH₂PO₄ 4.5 g, CaCl₂ (anhydrous) 1 g, MgSO₄·7H₂O 1 g, pH to 5.5 adjusted with 50% NaOH with Milli-Q H₂O to bring to 966.5 mL. After sterilization, the following were added: 26 mL 60% Glucose/Sophrose, and 400X *T. reesei* Trace Metals 2.5 mL.

[00247] The nucleotide sequence of the *Ate CA1* gene of pTTT-Ate CA1 is set forth as SEQ ID NO: 41. (The intron sequence is underlined).

ATGAAGCTCACTGCTGCCGTTCTCTCCCTGGCTGTGGCCGCCTCGGCCTCTTGCATCCGCCATGCCCGTC
GGGCTGACGGCGTCGTTGAGACCAACTCCTATAACTACACCGAGATGGGCGGTCCGCTGAACTGGTACGG
CCTGGACCCCGAGGCCAACTCTGCCTGCGCCACGGGCAAGCACCAGTCCCCCATCGTCATCCACTCCGAG
GACATCGACTATGTCTCCCCGGGATCCCTGAAGTTTCGACATCCCCAAGGCCGACTACGCCAAGTTTGAGA
ACCTTGGGTCCGGCCTCGAGGTCGTTCTGACCAACGGATCTCTCACTGTGGGCAACAAGAGCCTTCCCCCT
GGCCCAGTTCCACTTCCATACCCCCAGCGAGCACCAGCGTCAACGACGAGTACTATCCCATGGAGGTTTAC
TTTGTGTTCCAAAACAAGGGTATGCAGCGTCCCATGCACTCTACAAGTATCAACCCTAACATCGTATAGC
CAAAGACACCGCCGTCGTCGGCTTCTTCTTCCAGCTCTCCGAGCTCGGATACTCCGTCCCCCTGTTTCGAC
ACCATCTTCGACCACGTTCTCGAGATCGAGGAGCCTGGTGCCTTCACCCACACCGGGGAGATGGACTTCG
CCGGCCTGACCCACCACCTCTACATGCATGGCATCTACCAGTACTCTGGCTCCCTGACCACCCCTCCCTG
CTCCGAGGACGTCGCTGGTACCTGAGCACCAGCCCCCTGCCCCTGACCGTCCAGGACTACAACAAGGTC
AAGAAGGTGCTCAAGTACAACGCGCGCTACACACAGAACGCCCTGGGCGAGGACAACCTCCTCGAGGTGG
CGGCCAAGAGCCTCAACTAG

[00248] The amino acid sequence of the *Ate CA1* precursor is set forth as SEQ ID NO: 42. The predicted signal peptide sequence is shown in italics.

*MKLTA*AVLSLAVAASASCIRHARRADGVVETNSYNYTEMGGPLNWyGLDPEANSACATGKHQSP I V I H S E
D I D Y V S P G S L K F D I P K A D Y A K F E N L G S G L E V L T N G S L T V G N K S L P L A Q F H F H T P S E H R V N D E Y Y P M E V H
F V F Q N K A K D T A V V G F F F Q L S E L G Y S V P L F D T I F D H V L E I E E P G A F T H T G E M D F A G L T H H L Y M H G I Y Q Y S G
S L T T P P C S E D V A W Y L S T E P L P L T V Q D Y N K V K V L K Y N A R Y T Q N A L G E D N L L E V A A K S L N

[00249] The amino acid sequence of the mature form of *Ate CA1* is set forth as SEQ ID NO: 43.

SCIRHARRADGVVETNSYNYTEMGGPLNWyGLDPEANSACATGKHQSP I V I H S E D I D Y V S P G S L K F D I P K

ADYAKFENLGSGLLEVLTNGSLTVGNKSLPLAQFHFHTPSEHRVNDEYYPMEVHVFVQNKAKDTAVVGFF
 FQLSELGYSVPLFDTIFDHVLEIEEPGAFTHTGEMDFAGLTHHLYMHGIYQYSGSLTTPPCSEDVAWYLS
 TEPLPLTVQDYNKVKKVLKYNARYTQNALGEDNLLEVAAKSLN

[00250] The nucleotide sequence of the *Ate CA1* gene of pTTT-Ate CA1 without the intron sequence is set forth as SEQ ID NO: 44

atgaagctcactgctgcccgttctctccctggctgtggcgcgcctcggcctcttgcatccgccatgcccgctc
 gggctgacggcgctcggttgagaccaactcctataactacaccgagatggcggtccgctgaactggtacgg
 cctggaccccgaggccaactctgctgcgccacgggcaagcaccagtcccccatcgtcatccactccgag
 gacatcgactatgtctccccgggatccctgaagttcgacatcccccaaggccgactacgccaagtttgaga
 accttgggtccggcctcgaggtcggttctgaccaacggatctctcactgtgggcaacaagagccttccct
 ggccagttccacttccataccccccagcgagcaccgcgtcaacgacgagtactatcccatggagggttcac
 tttgtgttccaaaacaaggccaaagacaccgcccgtcgctcggttcttcttccagctctccgagctcgat
 actccgtccccctgttcgacaccatcttcgaccacgttctcgagatcgaggagcctggtgccttcaccca
 caccggggagatggacttcgcccgcctgaccaccacctctacatgcatggcatctaccagtactctggc
 tccctgaccacccctccctgctccgaggacgtcgccctggtacctgagcaccgagccccctgccccctgaccg
 tccaggactacaacaaggtcaagaaggtgctcaagtacaacgcgcgctacacacagaacgcctgggcga
 ggacaacctcctcgaggtggcgcccaagagcctcaactag

Purification of Ate CA1

[00251] Ate CA1 was purified from concentrated fermentation broth of a 7-L fermentor using three chromatography columns: 1) a phenyl sepharose column equilibrated with 20 mM Tris HCl, pH 8.0, containing 1 M ammonium sulfate, from which the protein was eluted using a linear gradient of the equilibration/wash buffer to 20 mM Tris HCl buffer, pH 8.0; 2) a DEAE sepharose column equilibrated with 20 mM Tris HCl buffer, pH 8.0, from which the protein was eluted using a linear gradient of equilibration/wash buffer to 20 mM Tris HCl buffer, pH 8.0, 0.5 M NaCl; and 3) a Superdex 75 gel filtration column, from which the protein was eluted using 20 mM sodium phosphate, pH 7.0, containing 0.15 M NaCl. The purified protein fractions were pooled and concentrated using a 3K Amicon Ultra-15 device, and the concentrated protein fraction was used in further studies.

C. Carbonic Anhydrase Activity of Ate CA1

[00252] The carbonic anhydrase activity of purified Ate CA1 was measured on ice in 20 mM Tris sulfate buffer (pH 8.3 at 25°C) containing 20 mM Na₂SO₄. Briefly, 3 mL of chilled 20 mM Tris sulfate buffer (pH 8.3 at 25°C) containing 20 mM Na₂SO₄ was added to a 50-mL polypropylene conical tube placed on ice. 20 µL of enzyme sample or buffer was added to the tubes followed by 2 mL of chilled CO₂ saturated water (purified and deionized using MilliQ Integral system, Millipore). After the contents were mixed, a standardized pH meter probe was immediately inserted into the solution and the time required for the pH to drop from 8.3 to 6.3 was recorded using a stop watch. The test was repeated until at least three blank measurements (with buffer added) were within a 15-second time window and the

average of three determinations ($T_{\text{Blank,avg}}$) was between 70 and 100 seconds. A standard curve was prepared using bovine carbonic anhydrase II (bCAII, Sigma C2522). The test measurements for determining activity were performed in triplicate, and $T_{\text{enzyme,avg}}$ was obtained.

[00253] One unit of activity (Wilbur-Anderson (W-A) unit, Wilbur and Anderson, Journal of Biological Chemistry 176:147-154, 1948) is defined as the time required for the pH of a 0.02 M Trizma buffer to drop from 8.3 to 6.3 per min at 0 °C.

$$\text{Units/mL enzyme} = (T_{\text{Blank,avg}} - T_{\text{enzyme,avg}}) * \text{DF} / (T_{\text{enzyme,avg}} * V)$$

where DF = dilution factor of enzyme sample, V = volume (in mL) of enzyme used, $T_{\text{Blank,avg}}$ = average time values of Blank measurements, and $T_{\text{enzyme,avg}}$ = average time values of Test measurements.

$$\text{Units/mg protein} = (\text{units/mL enzyme}) / (\text{mg protein/mL enzyme})$$

[00254] The specific activity of purified Ate CA1 was determined to be 1,327±285 units/mg using the above method. Thus, carbonic anhydrase activity of Ate CA1 is sufficient for enzymatic CO₂ sequestration processes.

D. Temperature Stability of Ate CA1

[00255] The temperature stability of Ate CA1 was determined in 0.1 M Tris buffer, pH 8.5 (adjusted with H₂SO₄ at 25°C), 0.1 M CHES buffer, pH 9.5 (adjusted with H₂SO₄ at 25°C), and 0.1 M CAPS buffer, pH 10.5 (adjusted with H₂SO₄ at 25°C). Na₂SO₄ was added to the buffer to a final concentration of 25 mM. 100 ppm of Ate CA1 in buffer, in 20 mL final volume, was incubated in a water bath at 40°C and 50°C. One sample was also incubated at room temperature (25°C). At different time points (5 minutes to 24 hours), 0.5 mL of the enzyme sample from each tube was taken and placed on ice. The specific activity of each enzyme sample was measured as described in Example 3C. The specific activity measurements were performed in triplicates. The percent remaining activity was calculated at each time point, and at each pH value. For each pH value, the activity of the sample kept at 0°C at time 0 was defined as 100% activity. Ate CA1 retained most of the activity under all pH conditions at 25°C and 40°C for 27 hrs. Also, Ate CA1 retained at least 60% activity at 50°C, pH 8.5 for 27 hrs, and retained about 50% activity at pH 9.5 for 3 hrs. As shown in Figures 12A-C, Ate CA1 is stable over different pH ranges typically used for enzymatic CO₂ extraction, and at supra-physiological temperatures.

E. Stability of Ate CA1 in 1 M NaHCO₃

[00256] The stability of Ate CA1 under high ionic strength conditions in the presence of bicarbonate was assessed. In a PCR machine, a 100 ppm solution of Ate CA1 diluted in 1 M NaHCO₃ was incubated at 20°C and 50°C. The specific activity of the sample was measured as described in Example 3C. Prior

to incubation and at varying time points (5 minutes to 3 hours), 100 μ L samples were withdrawn, cooled on ice and their specific activity measured in triplicate. The percent remaining activity was calculated for each time point. The activity of the sample kept on ice at time 0 was defined as 100% activity. Figure 13 shows stability of Ate CA1 in 1 M NaHCO_3 . Ate CA1 retains most of the activity over a 3-hr incubation period at both 20°C and 50°C. Thus, AteCA1 is stable in the presence of high concentrations of NaHCO_3 that are encountered in enzymatic CO_2 extraction.

F. Heat Capacity Measurement of Ate CA1

[00257] Excessive heat capacity curves were measured for Ate CA1 and bovine CAII (bCAII) using an ultrasensitive scanning high-throughput microcalorimeter, VP-Cap DSC (MicroCal, Inc., Northampton, MA). The standard procedure for differential scanning calorimetry (DSC) measurements and the theory of the technique has been previously described (Freire, *Methods Mol Biol*, 41:191-218, 1995). About 500 μ L of 0.5 mg/mL of each enzyme was studied. The proteins were scanned over a 35-100°C temperature range. The same sample was then re-scanned (following cooling) to check the reversibility of the thermal unfolding process. For both the carbonic anhydrases studied, thermal unfolding was irreversible. The proteins were studied in the following buffers: (1) 0.1 M Tris, pH 8.5, (2) 0.1 M CHES, pH 9.5, (3) 0.1 M CAPS, pH 10.5, and (4) 0.1 M CHES, pH 9.5 + 2 M NaCl. A 200°C/hr scan rate was used to minimize any artifacts that might have resulted from aggregation. The apparent thermal midpoint ($T_m(\text{app})$) of the DSC curves was used as an indicator of thermal stability. The melting points for Ate CA1 and bCAII are shown in Table 3F-1.

[00258] The thermal unfolding $T_m(\text{app})$ values for the carbonic anhydrase proteins showed a dependence on pH over the range of 8.5 to 10.5. Bovine carbonic anhydrase II has highest $T_m(\text{app})$ values between pH 8.5 and pH 9.5; Ate CA1 had the highest $T_m(\text{app})$ values at pH 8.5. The 0.1 M CHES buffer, pH 9.5 with 2 M NaCl decreased the $T_m(\text{app})$ for bCA II. For Ate CA1, the 0.1 M CHES, pH 9.5 buffer with 2 M NaCl increased the $T_m(\text{app})$ by about 7°C, as compared to the effect of 0.1 M CHES buffer, pH 9.5, with no additives. Thus, Ate CA1 is suitable for enzymatic CO_2 extraction at higher temperatures because the T_m of this enzyme increases under high ionic strength conditions typical of enzymatic CO_2 extraction.

Table 3F-1: Melting point temperatures for Ate CA1 and bCAII

	Buffers			
	#1	#2	#3	#4
Protein	0.1 M Tris, pH 8.5	0.1 M CHES, pH 9.5	0.1 M CAPS, pH 10.5	0.1 M CHES, pH 9.5 + 2 M NaCl
Bovine CA II	68.8	69.4	66.6	61.7
Ate CA1	67.8	63.9	60.8	71.4

[00259] $T_m(\text{app})$ was also determined for bCAII and Ate CA1 in 1 M KHCO_3 with no added NaCl, at various concentrations of added NaCl, ranging from 0.1 M to 1 M. As shown in Table 3F-2, $T_m(\text{app})$ decreased for bCAII in bicarbonate solution as compared to $T_m(\text{app})$ in buffer, while $T_m(\text{app})$ increased for Ate CA1 in bicarbonate solution, and with increased ionic strength. This increase in $T_m(\text{app})$ indicated the suitability of the enzyme Ate CA1 for enzymatic CO_2 extraction, especially in high ionic strength and in the presence of high concentrations of carbonate solutions. A higher $T_m(\text{app})$ was observed for Ate CA1 at higher bicarbonate concentrations as compared to $T_m(\text{app})$ at lower bicarbonate concentrations, which was unexpected.

Table 3F-2: $T_m(\text{app})$ [$^{\circ}\text{C}$] of Ate CA1 in 1 M KHCO_3

[Salt] (M)	Bovine CA II	Ate CA1
0	62.1	70.4
0.1	62.8	71.8
0.5	62.5	73.1
1	62.7	74.3

G. Homology Identification

[00260] The Ate CA1 mature protein sequence (267 residues) was subjected to a BLASTP search, and four protein sequences were identified with 50% or greater identity. Figure 14 shows an alignment of Ate CA1 with similar carbonic anhydrases sequences generated with Vector NTI (Invitrogen). Table 3G-1 shows the percent identities and NCBI Accession Nos. of the aligned sequences: XP_001827551 (SEQ ID NO: 45), XP_002148277 (SEQ ID NO: 46), XP_002152231 (SEQ ID NO: 47), and XP_002384772 (SEQ ID NO: 48). The aligned sequences were used to prepare a phylogenetic tree using program MEGA 5. Figure 15 shows the phylogenetic tree for Ate CA1 and its homologs.

Table 3G-1: Percent Identity Shared by Ate CA1 and Similar Sequences

Homolog	SEQ ID NO:	% IDENTITY (PID)
XP_001827551	45	67
XP_002148277	46	55
XP_002152231	47	56
XP_002384772	48	67

EXAMPLE 4

A. Sequence of *Streptomyces pristinaespiralis* Carbonic Anhydrase1 (Spr CA1)

[00261] *Streptomyces pristinaespiralis* was purchased from ATCC (ATCC Number 25486), and genomic DNA was isolated as described in Kieser et al., *Practical Streptomyces Genetics*, The John Innes Foundation, Norwich, United Kingdom (2000). The nucleic acid sequence for the *Spr CA1* gene (NCBI Reference Sequence P_06907409.1), and the amino acid sequence of the hypothetical protein encoded by the *Spr CA1* gene was found in the NCBI Databases (NCBI Accession No. ZP_06907409.1).

B. Expression of *Streptomyces pristinaespiralis* Carbonic Anhydrase1 (*Spr CA1*)

[00262] The *Spr CA1* gene was identified by genome annotation and amplified from genomic DNA of *Streptomyces pristinaespiralis* by PCR. The primers were designed based on the *Spr CA1* gene sequence in the public database. The primers used were: SprCA1-Fw 5'-AGCGCTAGCC GGCCCCCGG CACAGGCCTC CCCCAGTCCC GCGACGGCCC-3' (SEQ ID NO: 49), and SprCA1-RV 5'-TCCGGATCCT TATCAGGCGA CGGTGTGCACCAGC-3' (SEQ ID NO: 50).

[00263] The resulting PCR fragment of mature *Spr CA1* DNA was digested with *NheI* and *BamHI* and ligated into the pKB128 vector, and then digested with the same restriction enzymes to obtain the expression plasmid pJG187 (Figure 16). The pKB128 plasmid is a derivative of the pKB105 plasmid (as described in U.S. Patent Application Publication No. 2006/0154843) and is the source of the A4 promoter-CelA signal sequence. The ligation mixture was used to transform *E. coli* TOP10 chemically competent cells (Invitrogen Corp.) following the manufacturer's protocol. The transformed cells were plated on Luria Agar plates supplemented with 50 ppm ampicillin and incubated overnight at 37°C. Three transformants were picked from the plate and inoculated into 5 ml Luria Broth supplemented with 50 ppm ampicillin. Cultures were grown overnight at 37°C, plasmid DNA extracted and the correct sequence of the *Spr CA1* gene was confirmed by DNA sequencing. The pJG187 plasmid was then used to transform *S. lividans* TK23 derived protoplasts, as described in U.S. Patent Application Publication No. 2006/0154843. The transformation techniques used are described in Kieser et al., *Practical Streptomyces Genetics*, The John Innes Foundation, Norwich, United Kingdom (2000). Transformed cells were plated on R5 selection plates and incubated at 30°C for 3 days. One clone from the *Streptomyces* transformation plate was inoculated in TSG medium in shake flasks at 28°C for 3 days. Cultures were then transferred to a *Streptomyces* 2 Modified Medium (as described in U.S. Pat. Application Publication No. 2006/0154843) and incubated for an additional 4 days at 28°C.

[00264] TSG medium: 16 g BD Difco tryptone, 4 g BD Bacto soytone, 20 g Sigma caseine (hydrolysate), and 10 g potassium phosphate, dibasic, brought to 1 L. After autoclaving, 50% glucose was added to a final concentration of 1.5%.

[00265] R5 plates were prepared with the following: 206 g sucrose, 0.5 g K₂SO₄, 20.24 g MgCl₂, 20 g

glucose, 0.2 g Difco casamino acids, 10 g Difco yeast extracts, 11.46 g TES, 4 g L-Asp, 4 mL of trace elements, 44 g Difco agar, 20 mL 5% K₂HPO₄, 8 mL 5 M CaCl₂•2H₂O and 14 mL 1N NaOH were added to a final volume of 1 L after autoclaving. After 20 hours, a layer of thiostrepton (50 µg/mL final concentration) was plated on the top of the plates.

- 5 [00266] The nucleotide sequence of the *Spr CA1* gene of plasmid pJG187 (A4-celA-SprCA1) is set forth as SEQ ID NO: 51:

TCCCCCGGTCCCGCGACGGCCCGCCCGCCCGGGCACTCCCGCGCAGGCCCTGCGCGAGCTGGCGG
CCGGCAACCGCCGCTGGCGCACCTTCCGGCAGCAGCATCCGCACGAGAACTCGGCCGTGCGCGAGGAACT
GATATCCGGTCAGGAACCTTCGCCGTGGTCCTCGGCTGCATCGACTCGCGGGTGCCGCCGGAAGTGGTC
10 TTCGATCAGGGCCTCGGCGACCTGATGACCGTGCGCTCCGCCGGTGAGGTGCTCGACGAGGCGGTCTCTCG
GCAGCGTCGCGTACGGGGTACTGGAGCTGGACATCCCCCTGGTCATGGTGCTCGGTACACAGTCTCTCGG
AGCGGTGACGGCGGCGGTGCACGCGGAGGAGACCGGCGAGGAACTCCCCGCCACATCCAGTACATCGCC
GACCGCATACGGCCGGCCATAGACCACTCCCAGGAGGGCGCGGCGCGCTCGACTCCACGATACCCGCA
ATGTCCAGATGGTCACGCGGCTCCTCGCGCAGGAGCCCCGACCTCGCGGCGAGGATCGCGGCCGGGAAGCT
15 CGCGGTGCTCGGCGCACGCTACGAACTGAGCTCGCAGCTGGTGCACACCGTCGCCTGA

- [00267] The amino acid sequence of the *Spr CA1* precursor is set forth as SEQ ID NO: 52. The predicted native signal peptide is shown in italics.

MKNTPRTNSSVGSRRLLRAAVAGGALASGGLVWAGTPASASPGPATARRPRPGTPAQALRELAAGNRR
WRTFRQQHPHENSAREELISGQEPFAVVLGCIDSRVPELVFDQGLGLMTVRSAGEVLDEAVLGSVAY
20 GVLELDIPLVMVLGHQSCGAVTAAVHAEETGEELPAHIQYIADRI RPAIDHSQEGAARVDSTITRNVQMV
TRLLAQEPDLAARIAAGKLAVVGARYELSSQLVHTVA

- [00268] The amino acid sequence of the mature form of *Spr CA1* is set forth as SEQ ID NO: 53:

SPGPATARRPRPGTPAQALRELAAGNRRWRTFRQQHPHENSAREELISGQEPFAVVLGCIDSRVPELV
FDQGLGLMTVRSAGEVLDEAVLGSVAYGVLELDIPLVMVLGHQSCGAVTAAVHAEETGEELPAHIQYIA
25 DRIRPAIDHSQEGAARVDSTITRNVQMVTRLLAQEPDLAARIAAGKLAVVGARYELSSQLVHTVA

Protein purification of Spr CA1

- [00269] *Streptomyces lividans* cells expressing *Spr CA1* protein were grown in shake flasks under standard conditions. The *Spr CA1* protein was purified from fermentation broth using a phenyl sepharose column equilibrated with 20 mM Tris HCl buffer, pH 8.0, 1 M ammonium sulfate, from which the
30 protein was eluted with Milli-Q water. The purified protein fractions were pooled and concentrated using a 3K Amicon Ultra-15 device and the concentrated protein fraction was used in further studies.

C. Carbonic Anhydrase Activity of *Spr CA1*

- [00270] The carbonic anhydrase activity of purified *Spr CA1* was measured on ice in 20 mM Tris sulfate buffer (pH 8.3 at 25°C) containing 20 mM Na₂SO₄. Briefly, 3 mL of chilled 20 mM Tris sulfate
35 buffer (pH 8.3 at 25°C) containing 20 mM Na₂SO₄ was added to a 50-mL polypropylene conical tube

placed on ice. 20 μ L of enzyme sample or buffer was added to the tubes followed by 2 mL of chilled CO₂-saturated water (purified and deionized using Milli-Q Integral system, Millipore). After the contents were mixed, a standardized pH meter probe was immediately inserted into the solution and the time required for the pH to drop from 8.3 to 6.3 was recorded using a stop watch. The test was repeated until at least three blank measurements (with buffer added) were within a 15-second time window and the average of three determinations ($T_{\text{Blank,avg}}$) was between 70 and 100 seconds. A standard curve was prepared using bovine carbonic anhydrase II (bCAII, Sigma C2522). The test measurements for determining activity were performed in triplicate, and $T_{\text{enzyme,avg}}$ was obtained.

[00271] One unit of activity (Wilbur-Anderson or W-A unit, Wilbur and Anderson, *Journal of Biological Chemistry* 176, 147-154, 1948) is defined as the time required for the pH of a 0.02 M Trizma buffer to drop from 8.3 to 6.3 per min at 0°C:

$$\text{Units/mL enzyme} = (T_{\text{Blank,avg}} - T_{\text{enzyme,avg}}) * \text{DF} / (T_{\text{enzyme,avg}} * V)$$

where DF = dilution factor of enzyme sample, V = volume (in mL) of enzyme used, $T_{\text{Blank,avg}}$ = average time values of Blank measurements, and $T_{\text{enzyme,avg}}$ = average time values of Test measurements.

$$\text{Units/mg protein} = (\text{units/mL enzyme}) / (\text{mg protein/mL enzyme})$$

[00272] The specific activity of purified Spr CA1 was determined to be 2,433 $\pm$ 412 units/mg using the above method. Carbonic anhydrase activity of Spr CA1 is suitable for enzyme based CO₂ extraction.

D. Temperature Stability of Spr CA1

[00273] The temperature stability of Spr CA1 was determined in 0.1 M Tris buffer, pH 8.5 (adjusted by H₂SO₄ at 25°C), 0.1 M CHES buffer, pH 9.5 (adjusted by H₂SO₄ at 25°C), and 0.1 M CAPS buffer, pH 10.5 (adjusted by H₂SO₄ at 25°C). Na₂SO₄ was added to the buffer to a final concentration of 25 mM. 100 ppm of Spr CA1 in 20 mL buffer was incubated in a water bath at 40°C and 50°C. One sample was also incubated at room temperature (25°C). At different time points (5 minutes to 72 hours), 0.5 mL of the enzyme sample from each tube was taken and placed on ice. The specific activity of the enzyme sample was measured as described in Example 4C. The specific activity measurements were performed in triplicates. The percent remaining activity was calculated at each time point, and at each pH value. For each pH value, the activity of the sample kept at 0°C at time 0 was defined as 100% activity. The Spr CA1 retained more than 60% activity over a 27-hr incubation period between 25°C and 40°C at pH 8.5 and pH 9.5. As shown in Figures 17A-C, Spr CA1 is stable over different pH ranges typically used for enzymatic CO₂ extraction, and at supra-physiological temperatures.

E. Stability of Spr CA1 in 1 M NaHCO₃

[00274] The stability of Spr CA1 under high ionic strength conditions in the presence of bicarbonate was assessed. In a PCR machine, a 100 ppm solution of Spr CA1 in 1 M NaHCO₃ was incubated at 20°C and 50°C. The specific activities of the samples were measured prior to incubation as described in Example 4C. At varying time points (5 minutes to 3 hours), 100 µL samples were withdrawn, cooled on ice and their specific activity measured. The percent remaining activity was calculated for each time point. The activity of the sample kept on ice at time 0 was defined as 100% activity. Figure 18 shows stability of Spr CA1 in 1 M NaHCO₃. Spr CA1 retains more than 60% of the activity over a 2-hr incubation period at both 20°C and 50°C. Thus, Spr CA1 is stable in the presence of high concentrations of NaHCO₃ that are encountered in enzymatic CO₂ extraction.

F. Heat Capacity Measurement of Spr CA1

[00275] Excessive heat capacity curves are measured for Spr CA1 and bovine CAII (bCAII) using an ultrasensitive scanning high-throughput microcalorimeter, VP-Cap DSC (MicroCal, Inc., Northampton, MA). The standard procedure for differential scanning calorimetry (DSC) measurements and the theory of the technique has been previously described (Freire, *Methods Mol Biol*, 41:191-218, 1995). Approximately 500 µL of 0.5 mg/mL of each enzyme is studied. The proteins are scanned over a 35-100°C temperature range. The same sample is then re-scanned (following cooling) to check the reversibility of the thermal unfolding process. The proteins are studied in the following buffers: (1) 0.1 M Tris, pH 8.5, (2) 0.1 M CHES, pH 9.5, (3) 0.1 M CAPS, pH 10.5, and (4) 0.1 M CHES, pH 9.5 + 2 M NaCl. A 200°C/hr scan rate is used to minimize any artifacts that might have resulted from aggregation. The apparent thermal midpoint (T_m(app)) of the DSC curves is used as an indicator of thermal stability

G. Homology Identification

[00276] The Spr CA1 mature protein sequence (216 residues) was subjected to a BLASTP search, and the top eight protein sequences were selected for sequence alignment (Vector NTI, Invitrogen). Figure 19 shows an alignment of Spr CA1 with similar carbonic anhydrase sequences. Table 4G-1 shows the percent identities and NCBI Accession Nos. of the aligned sequences: YP_003487983 (SEQ ID NO: 54), ZP_04697556 (SEQ ID NO: 55), ZP_04996739 (SEQ ID NO: 56), ZP_05006186 (SEQ ID NO: 57), ZP_06588467 (SEQ ID NO: 58), ZP_06769567 (SEQ ID NO: 59), ZP_07290742 (SEQ ID NO: 60), and ZP_07308563 (SEQ ID NO: 61). The aligned sequences were used to prepare a phylogenetic tree using the program MEGA 5 (see, Tamura K. et al. *Molecular Biology and Evolution* (2011, in press). Figure 20 shows the phylogenetic tree for Spr CA1 and its homologs.

Table 4G-1: Percent Identity Shared by Spr CA1 and Similar Sequences

Homolog	SEQ ID NO:	% IDENTITY (PID)
YP_003487983	54	61
ZP_04697556	55	66
ZP_04996739	56	63
ZP_05006186	57	68
ZP_06588467	58	69
ZP_06769567	59	54
ZP_07290742	60	64
ZP_07308563	61	60

EXAMPLE 5**5 A. Cloning of *Bacillus agaradhaerens* carbonic anhydrase *Bag CA1***

[00277] The *Bacillus agaradhaerens* strain was selected as a potential source for various enzymes, useful for industrial applications. The entire genome of the *Bacillus agaradhaerens* strain was sequenced using Illumina's next generation sequencing technology. Genomic DNA for sequencing was obtained by first growing *Bacillus agaradhaerens* on Heart Infusion agar plates (Difco) at 37°C for 24 h. Cell material was scraped from the plates and used to prepare genomic DNA with the ZF Fungal/Bacterial DNA miniprep kit from Zymo (Cat No. D6005). This genomic DNA was used for genome sequencing and to amplify the *Bag CA1* gene for expression cloning. Genome sequencing and assembly of the sequence data was performed by BaseClear (Leiden, The Netherlands). Contigs were annotated by BioXpr (Namur, Belgium). One of genes identified this way in *Bacillus agaradhaerens* encodes a carbonic anhydrase that showed homology to carbonic anhydrases of various organisms. The sequence of this gene, called the *BagCA1* gene, is depicted in SEQ ID NO: 62. The protein encoded by the *Bag CA1* gene is depicted in SEQ ID NO: 63. The gene has an alternative start codon (GTG). At the N-terminus, the protein has a signal peptide with a length of 39 amino acids as predicted by SignalP-NN (Emanuelsson et al., Nature Protocols, 2:953-971, 2007). The presence of a signal sequence suggests that *BagCA1* is a secreted enzyme.

[00278] The nucleotide sequence of the *Bag CA1* gene isolated from *Bacillus agaradhaerens* is set forth as SEQ ID NO: 62. The sequence encoding the predicted native signal peptide is shown in *italics*:

GTGAAAAATGACGTCATGAAAGAAGGAACAAACATGAAAAGAAGAGTAGGGTGGGGAAGC
GTTGTAGTCGTTTTAGTGTCAAGTGTCGTCATAACGGCCTGTTCTACAGCGCAATCAGGA
GACGAAGAAGTTAACTCATTAAATGGCAGTGGCACTCATGAAGAGGCAATAAAAGACGAA
CATGAGGGGGCATTGGTCATATACTGGAGAACTGGACCGACGCATTGGGGATCGTTAGAC
GCCTCTTATGAATTATGTGAACAAGAGCAGGAACAATCGCCGATCAACATTGAGACAGAT
GAGGTGACAACACTACTGATACTCATATCAGCATCGCGTATCAACCGAGCCCGTTTGCGATC

GAAAATAACGGTCATACGATTCAAGCCAATGCCCTAACAGAGGATAATACTATCTCGATA
 GAGGGTGAGAATTATCAATTAATTCAATTTCACTTCCATGTCCCTTCTGAACATCAAAAA
 AATGGAGAACACTTAGACATGGAGCTTCATTTTGTCCATCAAAATCAAGAGGGGGAGTTG
 GCAGTGCTGGGTGTCCTAATGGAAGAAGGGGAGGTGAACGACGCATTAGCAGAGCTATGG
 5 GCTGAAATGCCACAAGAAGAGATGGATGAAACGATTGAATTAACGGATGCTATCGATCTT
 AACGCATTATTGCCAAGCAGCCATGAAGGCTTTCATTATGGTGGTTCTCTTACAACGCCT
 CCTTGTACTGAAGGTGTAAAATGGGTTCGTCCTCGAAAAACAATTTCCGTCTCGCAAGAA
 CAAATTGACACATTTCGAGAGATCTTTCCAACCAATAATCGGCCTGTCCAACCGTGGAAT
 GACCGGCATGTATATGAAGTGGCTATTGAT

10

[00279] The amino acid sequence of the Bag CA1 precursor protein is set forth as SEQ ID NO: 63.
 The predicted native signal peptide is shown in *italics*.

*VKNDVMKEGTNMKRRV*GWGSVVVVLVSSVVITACSTAQSGDEEVNSLNGSGTHEEAIKDE
 HEGHWSYTGETGPTHWGSLDASYELCEQEQQSPINIETDEVTTTDTTHISIAYQSPFAI
 15 ENNGHTIQANALTEDNTISIEGENYQLIQFHFHVPSEHQKNGEHLDMELHFVHQNEGEL
 AVLGVLMEEGEVNDALAEWAEMPQEEMDETIELTDAIDLNALLPSSHEGFHYGGSLLTP
 PCTEGVKWVVLEKTISVSQEQIDTFAEIFPTNNRPVQPWNDRHVYEVAID

[00280] The amino acid sequence of the mature form of BagCA1 is set forth as SEQ ID NO:64:

GDEEVNSLNGSGTHEEAIKDEHEGHWSYTGETGPTHWGSLDASYELCEQEQQSPINIET
 DEVTTTDTTHISIAYQSPFAIENNGHTIQANALTEDNTISIEGENYQLIQFHFHVPSEHQ
 20 KNGEHLDMELHFVHQNEGELAVLGVLMEEGEVNDALAEWAEMPQEEMDETIELTDAID
 LNALLPSSHEGFHYGGSLLTPCTEGVKWVVLEKTISVSQEQIDTFAEIFPTNNRPVQPW
 NDRHVYEVAID

25

B. Expression of *Bacillus agaradhaerens* Carbonic Anhydrase (*Bag CA1*)

[00281] The *Bag CA1* gene was amplified from genomic DNA of *Bacillus agaradhaerens* by PCR.
 PCR was performed using a thermocycler with KOD-plus polymerase (TOYOKAI) according to the
 instructions of the manufacturer (annealing temperature of 59°C). The primers used were: Bag CA1-Fw
 5'- GCG GCTAGC GCA GGAGACGAAGAAGTTAACTCAT -3' (SEQ ID NO: 65), and Bag CA1-Rv
 30 5'- TCA tta ATCAATAGCCACTTCATATACATG -3' (SEQ ID NO: 66).

[00282] The resulting PCR fragment of mature *Bag CA1* DNA was digested with NheI (the product
 from KOD-plus is blunt end) and ligated using T4 DNA ligase into pHPLT02 vector (50 ng/μL) digested
 with NheI and HpaI (blunt end) to obtain the expression plasmid pHPLT02-Bag CA1 (Figure 21). The
 reaction conditions used for ligation were according to the instructions of the supplier (New England
 35 Biolabs, MA). The pHPLT02 vector contains the thermostable amylase LAT promoter (pLAT) and a
 signal peptide (SEQ ID NO: 73) from *Bacillus licheniformis* strain DSM13 for expression of Bag CA1.
 The vector can replicate in *B. subtilis*. The ligation mixture was amplified using a rolling circle kit (GE
 Healthcare Life Sciences, NJ) and *B. subtilis* cells (degUHy32, ΔnprB, Δvpr, Δepr, ΔscoC, ΔwprA,
 Δmpr, ΔispA, Δbpr) were transformed with the amplified ligation mixture. The transformed cells were
 40 plated on Luria Agar plates supplemented with 10 ppm kanamycin. About 50-100 colonies were
 obtained, 24 of which were picked and grown in 24-well plates. The sequence of *Bag CA1* gene was
 confirmed by DNA sequencing. Selected clones from the 24 well plates were further grown in a 7L

fermentor.

[00283] The nucleotide sequence of the *Bag CA1* gene in plasmid pHPLT02-Bag CA1 is set forth as SEQ ID NO: 67. The signal sequence is shown in italics:

5 *ATGGTCTTTAAAAACCGAAAGTCTTTATCGCAGCGGTCATCCTGGCGCTGAGCAGCTTT*
GCGGGAACGGCAGCTAGCGCAGGAGACGAAGAAGTTAACTCATTAATGGCAGTGGCACT
CATGAAGAGGCAATAAAAGACGAACATGAGGGGCATTGGTCATATACTGGAGAACTGGA
CCGACGCATTGGGGATCGTTAGACGCCTCTTATGAATTATGTGAACAAGAGCAGGAACAA
TCGCCGATCAACATTGAGACAGATGAGGTGACAACACTACTGATACTCATATCAGCATCGCG
10 TATCAACCGAGCCCGTTTGGCGATCGAAAATAACGGTCATACGATTCAAGCCAATGCCCTA
ACAGAGGATAATACTATCTCGATAGAGGGTGAGAATTATCAATTAATTCAATTTCACTTC
CATGTCCCTTCTGAACATCAAAAAAATGGAGAACACTTAGACATGGAGCTTCATTTTGTC
CATCAAAATCAAGAGGGGGAGTTGGCAGTGCTGGGTGTCCTAATGGAAGAAGGGGAGGTG
AACGACGCATTAGCAGAGCTATGGGCTGAAATGCCACAAGAAGAGATGGATGAAACGATT
15 GAATTAACGGATGCTATCGATCTTAACGCATTATTGCCAAGCAGCCATGAAGGCTTTCAT
TATGGTGGTTCTCTTACAACGCCTCCTTGTACTGAAGGTGTAAAATGGGTGTCCTCGAA
AAAACAATTTCCGTCTCGCAAGAACAAATTGACACATTCGCAGAGATCTTTCCAACCAAT
AATCGGCCTGTCCAACCGTGGAATGACCGGCATGTATATGAAGTGGCTATTGAT

20 **[00284]** The amino acid sequence of the Bag CA1 precursor protein expressed from plasmid pHPLT02-Bag CA1 is set forth as SEQ ID NO: 68. The signal sequence is shown in italics:

25 *MVFKKPKVFIAAVILALSSFAGTAASAGDEEVNSLNGSGTHEEAIKDEHEGHWSYTGETGPTHWGS*
LDASYELCEQEQQSPINIETDEVTITDTHISIA YQSPFAIENNGHTIQANALTEDNTISIEGENYQ
LIQFHFHVPSEHQKNGEHLDMELHFVHQNQEGELAVLGVLMEEGEVNDALAEWAEMPQEEM
DETIELTDAIDLNALLPSSHEGFHYGGSLTTPPCTEGVKWVVLEKTISVSQEIQIDTFAEIFPTNNRP
VQPWNDRHVYEVAID

30 **[00285]** Several signal sequences from *Bacillus licheniformis* were used to express Bag CA1. These sequences are listed in Table 5B-1.

Table 5B-1: Signal sequences used to express Bag CA1

SEQ ID	Signal Sequence (SS)
69	MKMWMRKALVALFTIATFGLVSPPAASA
70	MNIKNIKKASALTVAALLAGGAPQASA
71	MKRKLMTLGLTAVLGSSAVLIPLKSNHASA
72	MKQQKRLYARLLPLLFALIFLLPHSAASA
73	MVFKKPKVFIAAVILALSSFAGTAASA

Protein purification of Bag CA1

35 **[00286]** Bag CA1 protein was purified from concentrated broth from a 7 L fermentor run using the two chromatography columns. 1) An anion exchange Q sepharose column equilibrated with 20 mM Tris-HCl pH 7.0 buffer from which the protein was eluted using a linear gradient of equilibration/wash buffer to 20 mM Tris-HCl, pH 7.0 buffer containing 0.5 M NaCl. 2) A phenyl sepharose column equilibrated

with 20 mM sodium phosphate, pH 6.0, containing 1 M ammonium sulfate from which the protein was eluted in the void volume. The purified protein fractions were pooled and concentrated using a 3K Amicon Ultra-15 device and the concentrated protein fraction was used in further studies.

5 C. Carbonic Anhydrase Activity of Bag CA1

[00287] The carbonic anhydrase activity of purified Bag CA1 was measured in 20 mM Tris sulfate buffer (pH 8.3 at 25°C) containing 20 mM Na₂SO₄. Briefly, 3 mL of chilled 20 mM Tris sulfate buffer (pH 8.3) containing 20 mM Na₂SO₄ was added to a 50-mL polypropylene conical tube placed on ice. Twenty (20) µL of enzyme sample or buffer was added to the tubes followed by 2 mL of chilled CO₂-saturated water (purified and deionized using Milli-Q Integral system, Millipore). After the contents were mixed, a standardized pH meter probe was immediately inserted into the solution and the time required for the pH to drop from 8.3 to 6.3 was recorded using a stop watch. The test was repeated until at least three blank measurements (with buffer added) were within a 15 sec time window and the average of three determinations ($T_{\text{Blank,avg}}$) was between 70-100 sec. A standard curve was prepared using bovine carbonic anhydrase II (bCAII, Sigma C2522). The test measurements for determining activity were performed in triplicate, and $T_{\text{enzyme,avg}}$ was obtained.

[00288] One unit of activity (One Wilbur-Anderson (W-A) unit, Wilbur and Anderson, *Journal of Biological Chemistry* 176, 147-154, 1948) is defined as the time required for the pH of a 0.02 M Trizma buffer to drop from 8.3 to 6.3 per min at 0°C:

$$\text{Units/mL enzyme} = (T_{\text{Blank,avg}} - T_{\text{enzyme,avg}}) * \text{DF} / T_{\text{enzyme,avg}} * V$$

where DF = dilution factor of enzyme sample, V = volume (in mL) of enzyme used, $T_{\text{Blank,avg}}$ = average time values of Blank measurements, and $T_{\text{enzyme,avg}}$ = average time values of Test measurements.

$$\text{Units/mL protein} = \text{units/mL enzyme} / \text{mg protein/mL enzyme}$$

[00289] The specific activity of purified Bag CA1 was determined to be 3715±274 units/mg using the above method. Carbonic anhydrase activity of Bag CA1 is suitable for enzyme based CO₂ extraction.

30 D. Temperature Stability of Bag CA1

[00290] The temperature stability of Bag CA1 was determined in 0.05 M Tris buffer, pH 8.5, (adjusted using H₂SO₄), 0.05 M CHES buffer, pH 9.5 (adjusted using H₂SO₄), and 0.05 M CAPS buffer, pH 10.5 (adjusted using H₂SO₄). BSA at a final concentration of 1 mg/mL and Na₂SO₄ at a final concentration of 25 mM were added to the buffers. One hundred (100) ppm of Bag CA1 diluted in 1 M NaHCO₃ was incubated in a water bath at 40°C and 50°C. One sample each was also incubated at room temperature (25°C) and on ice (0°C). At different time points (5 min to 24 h), 0.1 mL of the enzyme

sample from each tube was taken and placed on ice. The specific activity of the enzyme sample was measured as described in Example 5C. The percent remaining activity was calculated at each time point, at each pH value. For each pH value, the activity of the sample kept at 0°C at time 0 was defined as 100% activity. As shown in Figures 22A-C, Bag CA1 retained more than 90% activity over a 24 h incubation period at 25°C for all pH ranges tested. At higher pH levels, for example, at pH 9.5, after about 2 h of incubation at 50°C, the enzyme lost nearly all of its carbonic anhydrase activity. On the other hand, it can be seen that Bag CA1 is suitably stable for in a broad pH range to allow its use in typical enzymatic CO₂ extraction operations, and at supra-physiological temperatures.

E. Stability of Bag CA1 in 1M NaHCO₃

[00291] The stability of Bag CA1 was measured in 20 mM Tris sulfate buffer, pH 8.3 containing 20 mM Na₂SO₄ in the presence of 1 M NaHCO₃. One hundred (100) ppm of Bag CA1 diluted in 1 M NaHCO₃ was incubated at 20°C and 50°C in a PCR machine. The specific activity of the samples was measured prior to incubation as described in Example 5C. At varying time points (5 min to 3 h), 100 µL samples were withdrawn, cooled on ice and their specific activity measured as described in Example 5C. The percent remaining activity was calculated for each time point. The activity of the sample kept on ice at time 0 was defined as 100% activity. Figure 23 shows stability of Bag CA1 in 1 M NaHCO₃. Bag CA1 retained most of the activity over a 3 h incubation period at 20°C. When incubated at 50°C, a somewhat more rapid decrease in activity was observed, however, about 45% of the initial activity remained after 3 h of incubation at this temperature.

F. Heat Capacity Measurement of Bag CA1

[00292] Excessive heat capacity curves were measured for Bag CA1 and bovine carbonic anhydrase II (bCA II) using an ultrasensitive scanning high-throughput microcalorimeter, VP-Cap DSC (MicroCal, Inc., Northampton, MA). The standard procedure for DSC measurements and the theory of the technique has been described previously (Freire, Differential Scanning Calorimetry, *Methods Mol Biol*, 41:191-218, 1995). Approximately 500 µL of a 0.5 mg/mL sample of each enzyme was studied. The proteins were scanned over a 35-100°C temperature range. The same samples were then re-scanned (following cooling) to check the reversibility of the thermal unfolding process. For both carbonic anhydrases studied the thermal unfolding was irreversible. The proteins were studied in the following buffers: (1) 0.1 M Tris, pH 8.5, (2) 0.1 M CHES, pH 9.5, (3) 0.1 M CAPS, pH 10.5, and (4) 0.1 M CHES, pH 9.5 + 2 M NaCl. A 200°C/h scan rate was used to minimize any artifacts that may result from aggregation. The apparent thermal midpoint [$T_m(\text{app})$] of the DSC curves was used as an indicator of the thermal stability and melting points for these carbonic anhydrase molecules. The melting points of both enzymes in various buffers are shown in Table 5F-1.

[00293] The $T_m(\text{app})$ values during thermal unfolding for both the carbonic anhydrase polypeptides show a dependence on pH over the range of 8.5 to 10.5. Bovine carbonic anhydrase II has highest values between pH 8.5 and pH 9.5, while Bag CA1 has the highest values at pH 8.5. The 0.1 M CHES, pH 9.5

with 2 M NaCl buffer decreased the $T_m(\text{app})$ for bovine CA II, but increased the $T_m(\text{app})$ for Bag CA1. For Bag CA1, it was also observed that the inclusion of a carbonate buffer increased the $T_m(\text{app})$ substantially, and the 0.1M CHES, pH 9.5 with 2 M NaCl increased the T_m by approximately 16°C.

Moreover it has been observed that the carbonic anhydrase polypeptides had an increased melting temperature at higher ionic strength conditions, which was surprising. Thus, Bag CA1 is well suited for enzymatic CO₂ extraction at higher temperatures because the T_m of this enzyme increases in carbonate solution and at high ionic strength conditions typical of enzymatic CO₂ extraction.

[00294] $T_m(\text{app})$ was also determined for bCAII, and Bag CA1 in 1M KHCO₃ with and without 1M NaCl (Table 5F-2).

Table 5F-1: Melting Temperature for Bag CA1 and bCAII

	Buffers			
	#1	#2	#3	#4
Protein	0.1 M Tris, pH 8.5	0.1 M CHES, pH 9.5	0.1 M CAPS, pH 10.5	0.1 M CHES, pH 9.5 + 2 M NaCl
Bovine CA II	68.8	69.4	66.6	61.7
Bag CA1	51	43.1	42.7	59.1/80.9

Table 5F-2: $T_m(\text{app})$ [°C] of Bag CA1 in 1 M KHCO₃

[Salt] (M)	bCA II	Bag CA1
0	62.1	70.3
1	62.7	80.1

G. Homology Identification

[00295] The Bag CA1 mature protein sequence (251 residues) was subjected to a BLASTP search, and the top ten protein sequences were selected for sequence alignment (Vector NTI, Invitrogen). Table 5G-1 shows the percent identities and GENBANK Accession Nos. of the aligned sequences NP_241226 (SEQ ID NO:16), YP_004645315 (SEQ ID NO:74), ZP_04248491 (SEQ ID NO:25), YP_003428428 (SEQ ID NO:19), ZP_04209878 (SEQ ID NO:24), BAK17997 (SEQ ID NO:75), YP_176677 (SEQ ID NO:22), YP_419944 (SEQ ID NO:35), ZP_08403178 (SEQ ID NO:76), and YP_003944556 (SEQ ID NO:21). The aligned sequences were used to prepare a phylogenetic tree using the program MEGA 5 (Figure 24). The phylogenetic tree for Bag CA1 and its homologs is shown in Figure 25.

Table 5G-1: Percent Identity Shared by Bag CA1 and Similar Sequences

Homolog	SEQ ID NO:	% IDENTITY (PID)
NP_241226	16	64
YP_004645315	74	50
ZP_04248491	25	44
YP_003428428	19	51
ZP_04209878	24	45
BAK17997	75	50
YP_176677	22	48
YP_419944	35	40
ZP_08403178	76	41
YP_003944556	21	45

EXAMPLE 6**5 A. Identification and Expression of *Vibrio* sp. AND4 Carbonic Anhydrase1 (Vsp CA1)**

[00296] The gene encoding a carbonic anhydrase (Vsp CA1) from *Vibrio* sp. AND4 (as determined by BLAST analysis) was synthesized by Generay (Shanghai Generay Biotech Co., Ltd). The nucleic acid sequence for the *Vsp CA1* gene was determined to be at location 66128 to 66847 (*Vibrio* sp. AND4 1103602000595, whole genome shotgun sequence, NCBI Reference Sequence NZ_ABGR01000001.1) and the amino acid sequence of the protein encoded by the *Vsp CA1* gene was found in the NCBI Database (NCBI Accession ZP_02194066, (/locus_tag="AND4_05779").

[00297] The nucleotide sequence of the *Vsp CA1* gene synthesized is set forth as SEQ ID NO:77:

GCAAACCTGGGGATACAAAGGCGATCATGGCCCCGAAAAATTGGGGCGAATTTGCATCAGAAT
 GCGCAAAAGGCCAAAACCAAAGCCCGATTGATATCCAGTCAGTTACGGAGGCCAAAACCTGGA
 15 TAAACTGAATTTTCGATTATGAAGGCAAAGTTATTAGCCTGCTGAATAACGGCCATACACTGC
 AAACGAAACTGGAAGGCCAAAATACGCTGATGGTTGATGGCACAGAATTCACACTGAAACA
 GTTTCATTTTCATACGCCGTCAGAAAATCATGTCAACGGCAAAGAGTATCCGCTGGAAGCAC
 ATTTTGTGCATGCAGACAAAGCAGGCCATCTGGCAGTTGTTGCAGTTTTCTTTAAACTTGGC
 20 GGCGAAAATCCGGAACCTGGCGAAACTGCTGGCAAATATCCCGAAAAAAGATCAAGTTGTGG
 CAATTAAAGTTCCGTTTGTATGCAGATAGCCTTCTGCCGAACAATAAAGATTATTATAGATTC
 GACGGCAGCCTGACAACGCCGCCGTGCAGCGAAGGCGTTAGATGGCTGGTTATCAAAGAAA
 CGCAGACAATCAGCCCGGAACAAGTTACAGCATTACGAAAGCAATGGGCCATAATAACAG
 ACCGATTCAGCCGCTTAATTCAAGAATGATTAGAACACTTCAA

[00298] The *Vsp CA1* gene was digested with *NheI* and *HpaI* and ligated using T4 DNA ligase into pHPLT02 vector (50 ng/μL) digested with the same restriction enzymes to obtain the expression plasmid pHPLT02-Vsp CA1 (Figure 26). The reaction conditions used for ligation were according to the instructions of the supplier (New England Biolabs, MA). The pHPLT02 vector contains the thermostable amylase LAT promoter (pLAT) and a signal peptide (SEQ ID NO: 83) from *Bacillus licheniformis* strain DSM13 for expression of Vsp CA1. The vector can replicate in *B. subtilis*. The ligation mixture was

amplified using a rolling circle kit (GE Healthcare Life Sciences, NJ) and *B. subtilis* cells (degUHy32, ΔnprB, Δvpr, Δepr, ΔscoC, ΔwprA, Δmpr, ΔispA, Δbpr) were transformed with the amplified ligation mixture. The transformed cells were plated on Luria Agar plates supplemented with 10 ppm kanamycin. About 50-100 colonies were obtained, 24 of which were picked and grown in 24-well plates. The
 5 sequence of *Vsp CA1* gene was confirmed by DNA sequencing. Selected clones from the 24-well plates were further grown in a 7L fermentor.

[00299] The nucleotide sequence of the *Vsp CA1* gene of plasmid pHPLT02-*Vsp CA1* is set forth as SEQ ID NO: 78. The signal sequence is in italics:

10 *ATGCTGATCAACAAAAGCAAAAAATTTTCGTCTTTAGCTTTATCTTTGTCATGATGCTGAGCCTGAG*
CTTTGTCAACGGCGAAGTCGCTAGCGCAGCAAACTGGGGATACAAAGGCGATCATGGCCCGG
 AAAATTTGGGGCGAATTTGCATCAGAATGCGCAAAAGGCCAAAACCAAAGCCCGATTGATAT
 CCAGTCAGTTACGGAGGCCAAAAGTGGATAAACTGAATTTTCGATTATGAAGGCCAAAGTTATT
 AGCCTGCTGAATAACGGCCATACACTGCAAACGAACTGGAAGGCCAAAATACGCTGATGG
 15 TTGATGGCACAGAATTCACACTGAAACAGTTTTCATTTTCATACGCCGTCAGAAAATCATGTC
 AACGGCAAAGAGTATCCGCTGGAAGCACATTTTGTGCATGCAGACAAAGCAGGCCATCTGG
 CAGTTGTTGCAGTTTCTTTAACTTGGCGGCGAAAATCCGGAAGTGGCGAAAGTGGTGGCA
 AATATCCCGAAAAAAGATCAAGTTGTGGCAATTAAAGTTCCGTTTGATGCAGATAGCCTTCT
 GCCGAACAATAAAGATTATTATAGATTCGACGGCAGCCTGACAAACGCCGCCGTGCAGCGAA
 GGCGTTAGATGGCTGGTTATCAAAGAAACGCAGACAATCAGCCCCGGAACAAGTTACAGCAT
 20 TCACGAAAGCAATGGGCCATAATAACAGACCGATTCAGCCGCTTAATTCAAGAATGATTAG
 AACACTTCAA

[00300] The amino acid sequence of the *Vsp CA1* precursor protein expressed from plasmid pHPLT02-*Vsp CA1* is set forth as SEQ ID NO: 79. The signal sequence is in italics:

25 *MLINKSKKFFVFSFIFVMMLSLSFVNGEVASAANWGYKGDHGPENWGEFASECAKGQNQSPIDIQS*
VTEAKLDKLNFDYEGKVISLLNNGHTLQTKLEGKNTLMVDGTEFTLKQFHFHTPSENHVNGKE
 YPLEAHFVHADKAGHLAVVAVFFKLGGENPELAKLLANIPKKDQVVAIKVPFDADSLPNPKD
 YYRFDGSLTTPPCSEGVRWLVKETQTISPEQVTAFTKAMGHNNRPIQLNSRMIRTLQ
 30

[00301] The amino acid sequence of the mature form of *Vsp CA1* is set forth as SEQ ID NO:80:

ANWGYKGDHGPENWGEFASECAKGQNQSPIDIQSVTEAKLDKLNFDYEGKVISLLNNGHTLQTK
 KLEGKNTLMVDGTEFTLKQFHFHTPSENHVNGKEYPLEAHFVHADKAGHLAVVAVFFKLGGEN
 35 NPELAKLLANIPKKDQVVAIKVPFDADSLPNPKDYRFDGSLTTPPCSEGVRWLVKETQTISPE
 QVTAFTKAMGHNNRPIQLNSRMIRTLQ

[00302] Several signal sequences from *Bacillus licheniformis* were used to express *Vsp CA1*. These sequences are listed in Table 6A-1.

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Table 6A-1: Signal sequences used to express Vsp CA1

SEQ ID	Signal Sequence (SS)
NO:81	MKKKPLFRTFMCAALIGSLLAPVAASA
NO:82	MKNVLAVFVVLIFVLGAFGTSGPASA
NO:83	MLINKSKKFFVFSFIFVMMLSLSFVNGEVASA

Protein purification of Vsp CA1

[00303] Vsp CA1 protein was purified from concentrated broth from a 7 L fermentor run using the three chromatography columns. 1) An anion exchange Q sepharose column equilibrated with 20 mM Tris-HCl pH 7.0 buffer from which the protein was eluted in the void volume. 2) A phenyl sepharose column equilibrated with 20 mM sodium phosphate, pH 6.0, containing 1 M ammonium sulfate from which the protein was eluted using a linear gradient of equilibration/wash buffer to 20 mM sodium phosphate, pH 6.0. 3) A Superdex 75 gel filtration column from which the protein was eluted using 20 mM sodium phosphate, pH 7.0, containing 0.15 M NaCl. The purified protein fractions were pooled and concentrated using a 3K Amicon Ultra-15 device and the concentrated protein fraction was used in further studies.

B. Carbonic Anhydrase Activities of Vsp CA1

[00304] The carbonic anhydrase activity of purified Vsp CA1 was measured in 20 mM Tris sulfate buffer (pH 8.3 at 25°C) containing 20 mM Na₂SO₄. Briefly, 3 mL of chilled 20 mM Tris sulfate buffer (pH 8.3) containing 20 mM Na₂SO₄ was added to a 50-mL polypropylene conical tube placed on ice. Twenty (20) µL of enzyme sample or buffer were added to the tubes followed by 2 mL of chilled CO₂-saturated water (purified and deionized using Milli-Q Integral system, Millipore). After the contents were mixed, a standardized pH meter probe was immediately inserted into the solution and the time required for the pH to drop from 8.3 to 6.3 was recorded using a stop watch. The test was repeated until at least three blank measurements (with buffer added) were within a 15 sec time window and the average of three determinations ($T_{\text{Blank,avg}}$) was between 70 and 100 sec. A standard curve was prepared using bovine carbonic anhydrase II (bCAII, Sigma C2522). The test measurements for determining activity were performed in triplicate, and $T_{\text{enzyme,avg}}$ was obtained.

[00305] One unit of activity (One Wilbur-Anderson (W-A) unit, Wilbur and Anderson, *Journal of Biological Chemistry* 176, 147-154, 1948) is defined as the time required for the pH of a 0.02 M Trizma buffer to drop from 8.3 to 6.3 per min at 0°C:

$$\text{Units/mL enzyme} = (T_{\text{Blank,avg}} - T_{\text{enzyme,avg}}) * \text{DF} / T_{\text{enzyme,avg}} * V$$

where DF = dilution factor of enzyme sample, V = volume (in mL) of enzyme used, $T_{\text{Blank,avg}}$ = average time values of Blank measurements, and $T_{\text{enzyme,avg}}$ = average time values of Test measurements.

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$$\text{Units/mL protein} = \text{units/mL enzyme} / \text{mg protein/mL enzyme}$$

The specific activity of purified Vsp CA1 was determined to be 10028 ± 551 units/mg using the above method. Carbonic anhydrase activity of Vsp CA1 is suitable for enzyme based CO_2 extraction.

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C. Temperature Stability of Vsp CA1

[00306] The temperature stability of Vsp CA1 was determined in 0.05 M Tris buffer, pH 8.5, (adjusted by H_2SO_4), 0.05 M CHES buffer, pH 9.5 (adjusted using H_2SO_4), and 0.05 M CAPS buffer, pH 10.5 (adjusted using H_2SO_4). BSA at a final concentration of 1 mg/mL and Na_2SO_4 at a final concentration of 25 mM were added to the buffers. One hundred (100) ppm of Vsp CA1 diluted in 1 M NaHCO_3 was incubated at 25°C, 40°C and 50°C in a PCR machine. The specific activity of the samples was measured prior to incubation as described in Example 6B. At varying time points (5 min to 24 h), 100 μL samples were withdrawn, cooled on ice and their specific activity measured as described in Example 6B. The percent remaining activity was calculated at each time point, at each pH value. For each pH value, the activity of the sample kept at 0°C at time 0 was defined as 100% activity. As shown in Figures 27A-C, Vsp CA1 retained more than 50% activity over a 24 h incubation period at 25°C for pH 8.5 and pH 9.5. The activity drop exhibited more dramatically as pH increases at the incubation of 40°C and 50°C. At 50°C, Vsp CA1 completely lost its activity within 4 h at pH 8.5.

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D. Stability of Vsp CA1 in 1M NaHCO_3

[00307] The stability of Vsp CA1 was measured in 20 mM Tris sulfate buffer, pH 8.3, containing 20 mM Na_2SO_4 in the presence of 1 M NaHCO_3 . One hundred (100) ppm of the protein was diluted in 1 M NaHCO_3 and incubated at 20°C and 50°C in a PCR machine. The specific activity of the sample was measured prior to incubation as described in Example 6B. At varying time points (5 min to 3 h), 100 μL samples were withdrawn, cooled on ice and their specific activity measured as described in Example 6B. The percent remaining activity was calculated for each time point. The activity of the sample kept on ice at time 0 was defined as 100% activity. Figure 28 shows stability of Vsp CA1 in 1 M NaHCO_3 . Vsp CA1 retained 50% activity over a 1 to 3 hr incubation period at 20°C. At 50°C, the activity loss was more rapid, but even at that high temperature, the enzyme retained at least 50% of its activity after about 30 min.

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E. Heat Capacity Measurements of Vsp CA1

[00308] Excessive heat capacity curves were measured for Vsp CA1 and bovine carbonic anhydrase

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II (bCA II) using an ultrasensitive scanning high-throughput microcalorimeter, VP-Cap DSC (MicroCal, Inc., Northampton, MA). The standard procedure for DSC measurements and the theory of the technique has been described previously (Freire, Differential Scanning Calorimetry, *Methods Mol Biol*, 41:191-218, 1995). Approximately 500 μ L of a 0.5 mg/mL sample of each enzyme was studied. The proteins were scanned over a 35-100°C temperature range. The same samples were then re-scanned (following cooling) to check the reversibility of the thermal unfolding process. For the Vsp CA1 carbonic anhydrase studied the thermal unfolding was irreversible. The proteins were studied in the following buffers: (1) 0.1 M Tris, pH 8.5, (2) 0.1 M CHES, pH 9.5, (3) 0.1 M CAPS, pH 10.5, and (4) 0.1 M CHES, pH 9.5 + 2 M NaCl. A 200°C/h scan rate was used to minimize any artifacts that may result from aggregation. The apparent thermal midpoint [$T_m(\text{app})$] of the DSC curves was used as an indicator of the thermal stability and melting points for these carbonic anhydrase molecules. The melting points of both enzymes in various buffers are shown in Table 6E-1.

[00309] The $T_m(\text{app})$ values during thermal unfolding for the Vsp CA1 carbonic anhydrase protein shows a dependence on pH over the range of 8.5 to 10.5. Similar to bCAII, Vsp CA1 shows the highest values between pH 8.5 and pH 9.5. The 0.1 M CHES, pH 9.5, with 2 M NaCl buffer decreased the $T_m(\text{app})$ for bovine CA II, but did not affect the $T_m(\text{app})$ for Vsp CA1. The carbonate buffer lowered the $T_m(\text{app})$ for Vsp CA1.

[00310] $T_m(\text{app})$ was also determined for bCAII and Vsp CA1 in 1 M KHCO_3 with and without 1M NaCl (Table 6E-2). It was noted, surprisingly, that as the ionic strength increases, the $T_m(\text{app})$ of Vsp CA1 increases.

Table 6E-1: Melting Temperature for Vsp CA1 and bCAII

	Buffers			
	#1	#2	#3	#4
Protein	0.1M Tris, pH 8.5	0.1M CHES, pH 9.5	0.1M CAPS, pH 10.5	0.1M CHES, pH 9.5 + 2M NaCl
Bovine CA II	68.8	69.4	66.6	61.7
Vsp CA1	43.8/71.7	46/67.2	46.5	46/72.3

Table 6E-2: $T_m(\text{app})$ [°C] of Vsp CA1 in 1M KHCO_3

[Salt] (M)	bCA II	Vsp CA1
0	62.1	68.5
1	62.7	72.7

F. Homology Identification

[00311] The Vsp CA1 mature protein sequence (219 residues) was subjected to a BLASTP search, and the resulting 43 protein sequences were selected for sequence alignment (Vector NTI, Invitrogen) and phylogenetic tree generation excluding the protein sequences with 99%-100% identity with each others. Table 6F-1 shows the percent identities and NCBI Accession Nos. of the aligned sequences. The aligned sequences were used to prepare a phylogenetic tree using the program MEGA 5. Figure 29 shows the phylogenetic tree for Vsp CA1 and its homologs.

10 **Table 6F-1: Percent Identity Shared by Vsp CA1 and Similar Sequences**

Homolog	SEQ ID NO:	% IDENTITY (PID)
ZP_02194066_Vibrio_sp._AND4_VspCA1	84	100
ZP_01984408	85	86.19
ZP_06178017	86	84.1
ZP_01258509	87	79.08
ZP_06179499	88	77.41
ZP_04922188_Vibrio_sp._Ex25_VspECA1	89	77.87
ZP_08104131	90	72.77
ZP_05909537	91	69.62
NP_801012	92	69.62
ZP_05119338	93	71.43
YP_004250915	94	62.96
NP_763394	95	58.9
NP_936393	96	58.47
ZP_05886259	97	55.04
EGF40970	98	53.81
NP_799731	99	53.81
ZP_01259505	100	53.39
ZP_04921370_Vibrio_sp._Ex25_VspECA2	101	53.39
ZP_06179713	102	53.39
ZP_05718187	103	53.19
ZP_05720395	104	52.77
ZP_06032086	105	53.91

YP_131463	106	56.03
ADT89517	107	54.78
ZP_01221338	108	55.17
ZP_06041212	109	53.04
YP_004577581	110	54.04
ZP_01958038	111	51.91
AEA79886	112	51.49
ZP_04415512	113	51.91
ZP_04919557	114	51.49
EGS58855	115	51.06
NP_232672	116	51.49
EGS74119	117	51.06
ZP_04404214	118	51.06
ZP_06156315	119	52.97
ZP_04412368	120	50.64
ZP_01676897	121	51.06
ZP_01951018	122	51.06
ZP_01866792	123	52
YP_003610599	33	50.83
ZP_05879020	124	56.41
YP_004152175	125	51.58
YP_004391460	126	50.22

EXAMPLE 7

A. Identification and Expression of *Vibrio* sp. Ex25 Carbonic Anhydrase1 (VspE CA1)

- [00312] The amino acid sequence of a putative carbonic anhydrase (VspE CA1) from *Vibrio* sp. Ex25 was found in the NCBI Database (NCBI Accession ZP_04922188, /locus_tag="VEx25_A1217") by BLAST analysis. The location of the nucleic acid sequence for *VspE CA1* gene from a *Vibrio* sp. Ex25 scf_1101759099903 genomic scaffold, whole genome shotgun sequence (NCBI Reference Sequence: NZ_DS267817.1) was found to be from 79099 to 79818. The gene encoding VspE CA1 from *Vibrio* sp. Ex25 was synthesized by Generay (Shanghai Generay Biotech Co., Ltd).
- 10 [00313] The nucleotide sequence of the *VspE CA1* gene synthesized is set forth as SEQ ID NO:127:

GCATCATGGGGCTATGAAGGCTCACATGGCCCGGAACACTGGGGCGAATTTGCAAGCGAA
TGCAGCAAAGGACAAAATCAGTCACCGATTAACATCGTTTCAGCAGCGGAAGCCAAACTT
GACAACTGCAGTTCGATTACCATGGCAAGGCGATTAGCCTGCTGAACAATGGCCATACA

CTGCAACGCTCACTGGAGGGAGATAATACGCTTCTGATCGATGGCAATGCGTTCACGCTG
 AAACAGTTCATTTCATACGCGGAGCGAAAACCATGTGGATGGAAAAGAGTATCCGCTG
 GAAGCGCATTTTCGTTTCATGCAGATACAACAGGCCATCTGGCAGTCGTTGCAGTTTTCTTC
 CAAAGCGGCAAAGCAAATCCTGATCTGGCGAACTTCTTGCGAACATTCCGAGCAAGGAT
 5 CAGGCAGTCGAAATTAAGTCCGTTTGAAGCGGATGCGCTGCTGCCAAGGACAAAGCA
 TATTACAGATTCAATGGCTCACTGACGACACCGCCGTGCTCAGAAGGCGTGAGATGGCTG
 GTCATGAAAGAAGCACAGACGATCAGCCCTGAACAGATTAAAGCGTTCACGAAGGTCATG
 GGCGAGAACAACAGACCGATCCAACCGCTTAATGCAAGAATGGTTCTGATGCAACAT

- 10 **[00314]** The *VspE CA1* gene was digested with *NheI* and *HpaI* and ligated using T4 DNA ligase into pHPLT02 vector (50 ng/μL) digested with the same restriction enzymes to obtain the expression plasmid pHPLT02-VspE CA1 (Figure 30). The reaction conditions used for ligation were according to the instructions of the supplier (New England Biolabs, MA). The pHPLT02 vector contains the thermostable amylase LAT promoter (pLAT) and a signal peptide (SEQ ID NO: 132) from *Bacillus licheniformis*
- 15 strain DSM13 for expression of VspE CA1. The vector can replicate in *B. subtilis*. The ligation mixture was amplified using a rolling circle kit (GE Healthcare Life Sciences, NJ) and *B. subtilis* cells (degUHy32, ΔnprB, Δvpr, Δepr, ΔscoC, ΔwprA, Δmpr, ΔispA, Δbpr) were transformed with the amplified ligation mixture. The transformed cells were plated on Luria Agar plates supplemented with 10 ppm kanamycin. About 50-100 colonies were obtained, 24 of which were picked and grown in 24-
- 20 well plates. The sequence of *VspE CA1* gene was confirmed by DNA sequencing. Selected clones from the 24-well plates were further grown in a 7 L fermentor.

[00315] The nucleotide sequence of the *VspE CA1* gene of plasmid pHPLT02-VspE CA1 is set forth as SEQ ID NO: 128. The signal sequence is shown in *italics*:

25 *ATGAAACAACAAAAACGCCTGTATGCGAGACTGCTGCCGCTGCTGTTTGCCTGATCTTTCTGCTGCCGCACAGCG*
CAGCTAGCGCAGCATCATGGGGCTATGAAGGCTCACATGGCCCGAACACTGGGGCGAATTTGCAAGCGAATGC
AGCAAAGGACAAAATCAGTCACCGATTAACATCGTTTCAGCAGCGGAAGCCAACTTGACAACTGCAGTTCGAT
TACCATGGCAAGGCGATTAGCCTGCTGAACAATGGCCATACACTGCAACGTCCTGGAGGGAGATAATACGCTT
CTGATCGATGGCAATGCGTTCACGCTGAAACAGTTCATTTCATACGCGGAGCGAAAACCATGTGGATGGAAAA
GAGTATCCGCTGGAAGCGCATTTCTGTTTCATGCAGATACAACAGGCCATCTGGCAGTCGTTGCAGTTTTCTTCCAAA
 30 *GCGGCAAAGCAAATCCTGATCTGGCGAACTTCTTGCGAACATTCCGAGCAAGGATCAGGCAGTCGAAATTAAC*
TGCCGTTTGAAGCGGATGCGCTGCTGCCGAAGGACAAAGCATATTACAGATTCAATGGCTCACTGACGACACCGC
CGTGCTCAGAAGGCGTGAGATGGCTGGTCATGAAAGAAGCACAGACGATCAGCCCTGAACAGATTAAAGCGTTC
ACGAAGGTCATGGGCGAGAACAACAGACCGATCCAACCGCTTAATGCAAGAATGGTTCTGATGCAACAT

- [00316]** The amino acid sequence of the VspE CA1 precursor protein expressed from plasmid
- 35 pHPLT02-VspE CA1 is set forth as SEQ ID NO: 129. The signal sequence is shown in *italics*:

MKQQRRLYLPLLFALIFLLPHSAASAASWGYEGSHGPEHWGEFASECSKGQNPINIVSAAEAKLDKLQFDYHGK
AISLLNNGHTLQTSLEGNLTLIDGNAFTLKQHFHTPSENHVDGKEYPLEAHFVHADTTGHLAVVAVFFQSGKANPDL
AKLLANIPSKDQAVEIKLPFEADALLPKDKAYYRFNGSLTTPPCSEGVRLVMKEAQTSPEQIKAF TKVMGENNRPIQP
LNARMVLMQH

- 40 **[00317]** The amino acid sequence of the mature form of VspE CA1 is set forth as SEQ ID NO:130:

ASWGYEGSHGPEHWGEFASECSKGQNPINIVSAAEAKLDKLQFDYHGKAISLLNNGHTLQTSLEGNLTLIDGNAF
TLKQHFHTPSENHVDGKEYPLEAHFVHADTTGHLAVVAVFFQSGKANPDLAKLLANIPSKDQAVEIKLPFEADALLPK
DKAYYRFNGSLTTPPCSEGVRLVMKEAQTSPEQIKAF TKVMGENNRPIQLNARMVLMQH

[00318] Several signal sequences from *Bacillus licheniformis* were used to express VspE CA1.

These sequences are listed in Table 7A-1

Table 7A-1: Signal sequences used to express VspE CA1

SEQ ID	Signal Sequence (SS)
131	MKRHTVNLSLAMLVLGFLLSFSYASA
132	MKQQKRLYARLLPLLALIFLLPHSAASA

5 *Protein purification of VspE CA1*

[00319] VspECA1 protein was purified from concentrated broth from a 7 L fermentor run using the three chromatography columns. 1) An anion exchange Q sepharose column equilibrated with 20 mM Tris-HCl, pH 8.0 buffer from which the protein was eluted in the void volume. 2) An anion exchange Q sepharose column equilibrated with 20 mM Tris-HCl, pH 8.0 buffer from which the protein was eluted

10 using a linear gradient of equilibration/wash buffer to 20 mM Tris-HCl, pH 8.0, containing 0.25 M NaCl. 3) A Superdex 75 gel filtration column from which the protein was eluted using 20 mM sodium phosphate, pH 7.0, containing 0.15 M NaCl. The purified protein fractions were pooled and concentrated using a 3K Amicon Ultra-15 device and the concentrated protein fraction was used in further studies.

B. Carbonic Anhydrase Activities of VspE CA1

15 [00320] The carbonic anhydrase activity of purified VspE CA1 was measured in 20 mM Tris sulfate buffer, pH 8.3 at 25°C, containing 20 mM Na₂SO₄. Briefly, 3 mL of chilled 20 mM Tris sulfate buffer, pH 8.3, containing 20 mM Na₂SO₄ was added to a 50-mL polypropylene conical tube placed on ice. Twenty (20) µL of enzyme sample or buffer were added to the tubes followed by 2 mL of chilled CO₂ saturated water (purified and deionized using Milli-Q Integral system, Millipore). After the contents were

20 mixed, a standardized pH meter probe was immediately inserted into the solution and the time required for the pH to drop from 8.3 to 6.3 was recorded using a stop watch. The test was repeated until at least three blank measurements (with buffer added) were within a 15 sec time window and the average of three determinations ($T_{\text{Blank,avg}}$) was between 70 and 100 sec. A standard curve was prepared using bovine carbonic anhydrase II (bCAII, Sigma C2522). The test measurements for determining activity were

25 performed in triplicate, and $T_{\text{enzyme,avg}}$ was obtained.

[00321] One unit of activity (One Wilbur-Anderson (W-A) unit, Wilbur and Anderson, *Journal of Biological Chemistry* 176, 147-154, 1948) is defined as the time required for the pH of a 0.02 M Trizma buffer to drop from 8.3 to 6.3 per min at 0°C:

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$$\text{Units/mL enzyme} = (T_{\text{Blank,avg}} - T_{\text{enzyme,avg}}) * \text{DF} / T_{\text{enzyme,avg}} * V$$

where DF = dilution factor of enzyme sample, V = volume (in mL) of enzyme used, $T_{\text{Blank,avg}}$ = average time values of Blank measurements, and $T_{\text{enzyme,avg}}$ = average time values of Test measurements.

5

$$\text{Units/mL protein} = \text{units/mL enzyme} / \text{mg protein/mL enzyme}$$

The specific activity of purified VspE CA1 was determined to be 4621 ± 431 units/mg using the above method. Carbonic anhydrase activity of VspE CA1 is suitable for enzyme based CO_2 extraction.

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C. Stability of VspE CA1 in 1M NaHCO_3

[00322] The stability of VspE CA1 was measured in 20 mM Tris sulfate buffer, pH 8.3, containing 20 mM Na_2SO_4 in the presence of 1 M NaHCO_3 . One hundred (100) ppm of the protein was diluted in 1 M NaHCO_3 and incubated at 20°C and 50°C in a PCR machine. The specific activity of the sample was measured prior to incubation as described in Example 7B. At varying time points (5 min to 3 h), 100 μL samples were withdrawn, cooled on ice and their specific activity measured as described in Example 7B. The percent remaining activity was calculated for each time point. The activity of the sample kept on ice at time 0 was defined as 100% activity. Figure 31 shows stability of VspE CA1 in 1 M NaHCO_3 . VspE CA1 remains mostly active over a period of 3 h incubation and will likely to remain thus active well beyond this time point at 25°C . The carbonic anhydrase activity decreased more rapidly at a much higher temperature, 50°C , but much of the carbonic anhydrase activity was preserved within the first hour of incubation even at such a high temperature.

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D. Heat Capacity Measurements of VspE CA1

[00323] Excessive heat capacity curves were measured for VspE CA1 and bovine carbonic anhydrase II (bCA II) using an ultrasensitive scanning high-throughput microcalorimeter, VP-Cap DSC (MicroCal, Inc., Northampton, MA). The standard procedure for DSC measurements and the theory of the technique has been described previously (Freire, Differential Scanning Calorimetry, *Methods Mol Biol*, 41:191-218, 1995). Approximately 500 μL of a 0.5 mg/mL sample of each enzyme was studied. The proteins were scanned over a 35 - 100°C temperature range. The same samples were then re-scanned (following cooling) to check the reversibility of the thermal unfolding process. For the VspE CA1 carbonic anhydrase studied the thermal unfolding was irreversible. The proteins were studied in the following buffers: (1) 0.1 M Tris, pH 8.5, (2) 0.1 M CHES, pH 9.5, (3) 0.1 M CAPS, pH 10.5, and (4) 0.1 M CHES, pH 9.5 + 2 M NaCl. A 200°C/h scan rate was used to minimize any artifacts that may result from aggregation. The apparent thermal midpoint [$T_m(\text{app})$] of the DSC curves was used as an indicator of the thermal stability and melting points for these carbonic anhydrase molecules. The melting points of both enzymes in various buffers are shown in Table 7D-1.

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The $T_m(\text{app})$ values during thermal unfolding for the VspE CA1 carbonic anhydrase protein shows a dependence on pH over the range of 8.5 to 10.5. Similar to bCAII, VspE CA1 shows the highest values between pH 8.5 and 9.5. The 0.1 M CHES, pH 9.5 with 2 M NaCl buffer decreased the $T_m(\text{app})$ for bovine CA II, but did not affect the $T_m(\text{app})$ for VspE CA1. The carbonate significantly increased the $T_m(\text{app})$ for VspE CA1. $T_m(\text{app})$ was also determined for bCAII and VspE CA1 in 1M KHCO_3 with and without 1M NaCl (Table 7D-2). It was observed that the VspE CA1 had a higher $T_m(\text{app})$ at 1 M salt as compared to the $T_m(\text{app})$ at 0 M salt.

Table 7D-1: Melting Temperature for VspE CA1 and bCAII

	Buffers			
	#1	#2	#3	#4
Protein	0.1M Tris, pH 8.5	0.1M CHES, pH 9.5	0.1M CAPS, pH 10.5	0.1M CHES, pH 9.5 + 2M NaCl
Bovine CA II	68.8	69.4	66.6	61.7
VspE CA1	49.9	44.9/60.9	44.5	53.4/68.8

Table 7D-2: $T_m(\text{app})$ [$^{\circ}\text{C}$] of VspE CA1 in 1M KHCO_3

[Salt] (M)	bCA II	VspE CA1
0	62.1	49.6
1	62.7	57.9

EXAMPLE 8

A. Identification and Expression of *Vibrio sp. Ex25* Carbonic Anhydrase2 (VspE CA2)

[00324] The amino acid sequence of another putative carbonic anhydrase (VspE CA2) from *Vibrio sp. Ex25* was found in the NCBI Database (NCBI Accession ZP_04921370, /locus_tag="VEx25_A0096") by BLAST analysis. The location of the nucleic acid sequence for VspE CA2 gene from a *Vibrio sp. Ex25* scf_1101759099880 genomic scaffold, whole genome shotgun sequence (NCBI Reference Sequence: NZ_DS267810.1) was found to be from 94584 to 95303. The gene encoding VspE CA2 from *Vibrio sp. Ex25* was synthesized by Genaray (Shanghai Genaray Biotech Co., Ltd).

[00325] The nucleotide sequence of the VspE CA2 gene synthesized is set forth as SEQ ID NO:133:
 TCAGAGTGGGGCTATGGCAATGATAAACATGGCCCGGAACATTGGGGCGAAATTGCAAAGGATTGCGCGACAAC
 GAAAAACCAATCACCGATTAACATTGACAATCCGGCCGACGCCAAGCTGGAAGCCCTTAATCTGTCATATACAGG
 CCAGGTTATTGGCCTGACGAACAATGGCCATACACTGCAAGCTCAAGTGAACGGCAGAAACAGCTTCACAATCGA

TAGCGAAACGTTTGAGCTTCAGCAGTTTCACTTTCATACACCGAGCGAGAACCAGATCAAAGGCAGACAGTATCC
 GCTTGAAGCGCACTTTGTTTCATGCAAATGCCGACGGCGAACTGGCAGTGATTTTCAGTTATGTTTGATGCAGGCGA
 TCAGAATGCAGCACTGAGCAAGCTGATCAATGCAATTCGCGAGGAGAACCAAACGACGTTCTTTAAGGACACGTT
 TGAGATCAACGACCTGCTGCCGAAGACGGCAAATTATTACAGATTCAACGGCTCACTGACAACGCCGCCGTGTAG
 5 CGAAGGCGTCAGATGGTTCGTTCTGAAAGACACACAAACTGTCAAAGGACCAGGCAGCCAACTGATGGAAG
 TTATGGGCCAAAATAACAGACCGCTGCAACCGCTGAATGCGAGAGTTGTGCTTAGCAAT

[00326] The *VspE* CA2 gene was digested with *NheI* and *HpaI* and ligated using T4 DNA ligase into
 pHPLT02 vector (50 ng/μL) digested with the same restriction enzymes to obtain the expression plasmid
 10 pHPLT02-VspE CA2 (Figure 32). The reaction conditions used for ligation were according to the
 instructions of the supplier (New England Biolabs, MA). The pHPLT02 vector contains the thermostable
 amylase LAT promoter (pLAT) and a signal peptide from *Bacillus licheniformis* strain DSM13 (for
 example, one that is part of SEQ ID NO: 135) for expression of VspE CA2. The vector can replicate in
B. subtilis. The ligation mixture was amplified using a rolling circle kit (GE Healthcare Life Sciences,
 15 NJ) and *B. subtilis* cells (degUHy32, ΔnprB, Δvpr, Δepr, ΔscoC, ΔwprA, Δmpr, ΔispA, Δbpr) were
 transformed with the amplified ligation mixture. The transformed cells were plated on Luria Agar plates
 supplemented with 10 ppm kanamycin. About 50-100 colonies were obtained, 24 of which were picked
 and grown in 24-well plates. The sequence of *VspE* CA2 gene was confirmed by DNA sequencing.
 Selected clones from the 24-well plates were further grown in a 7 L fermentor.

[00327] The nucleotide sequence of the *VspE* CA2 gene of plasmid pHPLT02-VspE CA2 is set forth
 20 as SEQ ID NO: 134. The signal sequence is shown in *italics*.

*ATGGTCTTTAAAAAACC*GAAAGTCTTTATCGCAGCGGTCATCTGGCGCTGAGCAGCTTTGCGGGAACGGCAGCT
 AGCGCATCAGAGTGGGGCTATGGCAATGATAAACATGGCCCGGAACATTGGGGCGAAATTGCAAAGGATTGCGC
 GACAACGAAAAACCAATCACCGATTAACATTGACAATCCGGCCGACGCCAAGCTGGAAGCCCTTAATCTGTCATA
 25 TACAGGCCAGGTTATTGGCCTGACGAACAATGGCCATACACTGCAAGCTCAAGTGAACGGCAGAAACAGCTTCAC
 AATCGATAGCGAAACGTTTGAGCTTCAGCAGTTTCACTTTCATACACCGAGCGAGAACCAGATCAAAGGCAGACA
 GTATCCGCTTGAAGCGCACTTTGTTTCATGCAAATGCCGACGGCGAACTGGCAGTGATTTTCAGTTATGTTTGATGCA
 GGCGATCAGAATGCAGCACTGAGCAAGCTGATCAATGCAATTCCGCGAGGAGAACCAAACGACGTTCTTTAAGGA
 CACGTTTGAGATCAACGACCTGCTGCCGAAGACGGCAAATTATTACAGATTCAACGGCTCACTGACAACGCCGCC
 30 GTGTAGCGAAGGCGTCAGATGGTTCGTTCTGAAAGACACACAAACTGTCAAAGGACCAGGCAGCCAACTGA
 TGAAGTTATGGGCCAAAATAACAGACCGCTGCAACCGCTGAATGCGAGAGTTGTGCTTAGCAAT

[00328] The amino acid sequence of the VspE CA2 precursor protein expressed from plasmid
 pHPLT02-VspE CA2 is set forth as SEQ ID NO: 135. The signal sequence is shown in *italics*.

*MVFKKPKVFIAAVILALSS*FAGTAASASEWGYGNDKHGPEHWGEIAKDCATTKNQSPINIDNPADAKLEALNLSYTGQ
 35 VIGLTNNGHTLQAQVNGRNSFTIDSETFELQQFHFTPSENQIKGRQYPLEAHFVHANADGELAVISVMFDAGDQNA
 ALSKLINAI PQENQTTFKDTFEINDLLPKTANYRFRNGSLTPPCSEGVRFVFLKDTQTL SKDQAAKLMEVMGQNNR
 PLQPLNARVVLSN

[00329] The amino acid sequence of the mature form of VspE CA2 is set forth as SEQ ID NO:136:

SEWGYGNDKHGPEHWGEIAKDCATTKNQSPINIDNPADAKLEALNLSYTGQVIGLTNNGHTLQAQVNGRNSFTIDSE
 40 TFELQQFHFTPSENQIKGRQYPLEAHFVHANADGELAVISVMFDAGDQNAALSKLINAI PQENQTTFKDTFEINDLL
 PKTANYRFRNGSLTPPCSEGVRFVFLKDTQTL SKDQAAKLMEVMGQNNRPLQPLNARVVLSN

Protein purification of VspE CA2

[00330] VspECA2 protein was purified from concentrated broth from a 7L fermentor run using the three chromatography columns. 1) An anion exchange Q sepharose column equilibrated with 20 mM Tris-HCl, pH 8.0 buffer from which the protein was eluted using a linear gradient of equilibration/wash buffer to 20 mM Tris-HCl, pH 8.0, containing 0.25 M NaCl. 2) An anion exchange Q sepharose column equilibrated with 20 mM Tris-HCl, pH 8.0 buffer from which the protein was eluted using a linear gradient of equilibration/wash buffer to 20 mM Tris-HCl, pH 8.0, containing 0.3 M NaCl. 3) A Superdex 75 gel filtration column from which the protein was eluted using 20 mM sodium phosphate, pH 7.0, containing 0.15 M NaCl. The purified protein fractions were pooled and concentrated using a 3K Amicon Ultra-15 device and the concentrated protein fraction was used in further studies.

B. Carbonic Anhydrase Activities of VspE CA2

[00331] The carbonic anhydrase activity of purified VspE CA2 was measured in 20 mM Tris sulfate buffer (pH 8.3 at 25°C) containing 20 mM Na₂SO₄. Briefly, 3 mL of chilled 20 mM Tris sulfate buffer, pH 8.3, containing 20 mM Na₂SO₄ was added to a 50-mL polypropylene conical tube placed on ice. Twenty (20) µL of enzyme sample or buffer were added to the tubes followed by 2 mL of chilled CO₂ saturated water (purified and deionized using Milli-Q Integral system, Millipore). After the contents were mixed, a standardized pH meter probe was immediately inserted into the solution and the time required for the pH to drop from 8.3 to 6.3 was recorded using a stop watch. The test was repeated until at least three blank measurements (with buffer added) were within a 15 sec time window and the average of three determinations ($T_{\text{Blank,avg}}$) was between 70 and 100 sec. A standard curve was prepared using bovine carbonic anhydrase II (bCAII, Sigma C2522). The test measurements for determining activity were performed in triplicate, and $T_{\text{enzyme,avg}}$ was obtained.

[00332] One unit of activity (One Wilbur-Anderson (W-A) unit, Wilbur and Anderson, *Journal of Biological Chemistry* 176, 147-154, 1948) is defined as the time required for the pH of a 0.02 M Trizma buffer to drop from 8.3 to 6.3 per min at 0°C:

$$\text{Units/mL enzyme} = (T_{\text{Blank,avg}} - T_{\text{enzyme,avg}}) * \text{DF} / T_{\text{enzyme,avg}} * V$$

where DF = dilution factor of enzyme sample, V = volume (in mL) of enzyme used, $T_{\text{Blank,avg}}$ = average time values of Blank measurements, and $T_{\text{enzyme,avg}}$ = average time values of Test measurements.

$$\text{Units/ml protein} = \text{units/mL enzyme} / \text{mg protein/mL enzyme}$$

The specific activity of purified VspE CA2 was determined to be 3189±112 units/mg using the above method. Carbonic anhydrase activity of VspE CA2 is suitable for enzyme based CO₂ extraction.

C. Stability of VspE CA2 in 1M NaHCO₃

[00333] The stability of VspE CA2 was measured in 20 mM Tris sulfate buffer, pH 8.3, containing 20 mM Na₂SO₄ in the presence of 1 M NaHCO₃. One hundred (100) ppm of the protein was diluted in 1 M NaHCO₃ and incubated at 20°C and 50°C in a PCR machine. The specific activity of the sample was measured prior to incubation as described in Example 8B. At varying time points (5 min to 3 h), 100 µL samples were withdrawn, cooled on ice and their specific activity measured as described in Example 8B. The percent remaining activity was calculated for each time point. The activity of the sample kept on ice at time 0 was defined as 100% activity. Figure 33 shows stability of VspE CA2 in 1 M NaHCO₃. VspE CA2 remains mostly active over a 3 h incubation at 25°C, and can be expected to remain mostly active for an extended period of incubation. At 50°C, the loss of activity was more prominent and at a higher rate. However, at least 40% of activity remained after 30 minutes of incubation at such a high temperature, indicating a substantial thermostability in this enzyme.

D. Heat Capacity Measurements of VspE CA2

[00334] Excessive heat capacity curves were measured for VspE CA2 and bovine carbonic anhydrase II (bCA II) using an ultrasensitive scanning high-throughput microcalorimeter, VP-Cap DSC (MicroCal, Inc., Northampton, MA). The standard procedure for DSC measurements and the theory of the technique has been described previously (Freire, Differential Scanning Calorimetry, *Methods Mol Biol*, 41:191-218, 1995). Approximately 500 µL of a 0.5 mg/mL sample of each enzyme was studied. The proteins were scanned over a 35-100°C temperature range. The same samples were then re-scanned (following cooling) to check the reversibility of the thermal unfolding process. For the VspE CA2 carbonic anhydrase studied the thermal unfolding was irreversible. The proteins were studied in the following buffers: (1) 0.1 M Tris, pH 8.5, (2) 0.1 M CHES, pH 9.5, (3) 0.1 M CAPS, pH 10.5, and (4) 0.1 M CHES, pH 9.5 + 2 M NaCl. A 200°C/h scan rate was used to minimize any artifacts that may result from aggregation. The apparent thermal midpoint [$T_m(\text{app})$] of the DSC curves was used as an indicator of the thermal stability and melting points for these carbonic anhydrase molecules. The melting points of both enzymes in various buffers are shown in Table 8D-1.

[00335] The $T_m(\text{app})$ values during thermal unfolding for the VspE CA2 carbonic anhydrase protein shows a dependence on pH over the range of 8.5 to 10.5. VspE CA2 shows the highest values at pH 8.5. The 0.1 M CHES, pH 9.5 with 2 M NaCl buffer decreased the $T_m(\text{app})$ for bovine CA II, but increased the $T_m(\text{app})$ for VspE CA2 by 7°C. The carbonate slightly increased the $T_m(\text{app})$ for VspE CA2. $T_m(\text{app})$ was also determined for bCAII and VspE CA2 in 1M KHCO₃ with and without 1M NaCl (Table 8D-2). Surprisingly, VspE CA2 appeared to have a higher $T_m(\text{app})$ at 1 M bicarbonate than at 0 M bicarbonate.

Table 8D-1: Melting Temperature for VspE CA2 and bCAII

Buffers				
	#1	#2	#3	#4
Protein	0.1 M Tris, pH 8.5	0.1 M CHES, pH 9.5	0.1 M CAPS, pH 10.5	0.1 M CHES, pH 9.5 + 2 M NaCl
Bovine CA II	68.8	69.4	66.6	61.7
VspE CA2	76.8	72.4	67.3	76.3

Table 8D-2: T_m(app) [°C] of VspE CA2 in 1M KHCO₃

[Salt] (M)	bCA II	VspE CA2
0	62.1	74.1
1	62.7	79.7

5

[00336] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

10

CLAIMS

We claim:

1. A recombinant polypeptide comprising an amino acid sequence , wherein said amino acid sequence has carbonic anhydrase activity, wherein said amino acid sequence has a higher
5 thermostability and/or melting temperature at a higher ionic strength, and wherein said amino acid sequence is at least 70 % identical to the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 28, SEQ ID NO: 43, SEQ ID NO: 53, SEQ ID NO: 64, SEQ ID NO: 80, SEQ ID NO:130, or SEQ ID NO: 136.

2. A recombinant polypeptide comprising an amino acid sequence , wherein said amino acid sequence has carbonic anhydrase activity greater than 25% of the carbonic anhydrase activity when
10 incubated for 30 minutes or longer at a pH of from about 8 to about 10.5, and wherein said amino acid sequence is at least 70 % identical to the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 28, SEQ ID NO: 43, SEQ ID NO: 53, SEQ ID NO: 64, SEQ ID NO: 80, SEQ ID NO:130, or SEQ ID NO: 136.

3. The recombinant polypeptide of Claims 1 or 2, wherein the amino acid sequence that is at least 70% identical to the amino acid sequence of SEQ ID NO: 3.

15 4. The recombinant polypeptide of Claim 3, wherein the polypeptide retains greater than 70% of the carbonic anhydrase activity when incubated for 5 or more hours at a pH of 8.5 to 10.5.

5. The recombinant polypeptide of Claim 3 or 4, wherein the polypeptide retains at least 90% of the carbonic anhydrase activity when incubated for about 24 hours at a temperature of about 25°C.

20 6. The recombinant polypeptide of any one of Claims 3-5, wherein the polypeptide retains at least 90% of the carbonic anhydrase activity when incubated for about 24 hours at a temperature of about 40°C.

25 7. The recombinant polypeptide of any one of Claims 3-6, wherein the polypeptide retains at least 50% of the carbonic anhydrase activity when incubated for about 3 hours at a temperature of about 50°C.

8. The recombinant polypeptide of any one of Claims 3-7, wherein the polypeptide retains at least 25% of the carbonic anhydrase activity when incubated for about 3 hours at a temperature of about 50°C.

30 9. The recombinant polypeptide of any one of Claims 3-8, wherein the polypeptide retains at least 50% of the carbonic anhydrase activity when incubated for 1 to 3 hours at a temperature of from 20°C to 50°C under high ionic strength conditions.

10. The recombinant polypeptide of Claims 1 or 2, wherein the amino acid sequence that is at least 70% identical to the amino acid sequence of SEQ ID NO: 28.

11. The recombinant polypeptide of Claim 10, wherein the polypeptide retains greater than 70% of the carbonic anhydrase activity when incubated for 5 or more hours at a pH of 8.5 to 9.5.

5 12. The recombinant polypeptide of Claim 10 or 11, wherein the polypeptide retains at least 80% of the carbonic anhydrase activity when incubated for about 24 hours at a temperature of about 25°C.

10 13. The recombinant polypeptide of any one of Claims 10-12, wherein the polypeptide retains at least 90% of the carbonic anhydrase activity when incubated for about 24 hours at a temperature of about 40°C.

14. The recombinant polypeptide of any one of Claims 10-13, wherein the polypeptide retains at least 40% of the carbonic anhydrase activity when incubated for about 3 hours at a temperature of about 50°C.

15 15. The recombinant polypeptide of any one of Claims 10-14, wherein the polypeptide retains at least 25% of the carbonic anhydrase activity when incubated for about 3 hours at a temperature of about 40°C.

16. The recombinant polypeptide of any one of Claims 10-15, wherein the polypeptide retains at least 50% of the carbonic anhydrase activity when incubated for 1 to 3 hours at a temperature of from 20°C to 50°C under high ionic strength conditions.

20 17. The recombinant polypeptide of Claims 1 or 2, wherein the amino acid sequence that is at least 70% identical to the amino acid sequence of SEQ ID NO: 43.

18. The recombinant polypeptide of Claim 17, wherein the polypeptide retains greater than 70% of the carbonic anhydrase activity when incubated for 5 or more hours at a pH of 8.5 to 10.5.

25 19. The recombinant polypeptide of Claims 17 or 18, wherein the polypeptide retains at least 90% of the carbonic anhydrase activity when incubated for about 24 hours at a temperature of about 25°C.

20. The recombinant polypeptide of any one of Claims 17-19, wherein the polypeptide retains at least 80% of the carbonic anhydrase activity when incubated for about 24 hours at a temperature of about 40°C.

30

21. The recombinant polypeptide of any one of Claims 17-20, wherein the polypeptide retains at least 40% of the carbonic anhydrase activity when incubated for about 3 hours at a temperature of about 50°C.

5 22. The recombinant polypeptide of any one of Claims 17-21, wherein the polypeptide retains at least 20% of the carbonic anhydrase activity when incubated for about 3 hours at a temperature of about 50°C.

23. The recombinant polypeptide of any one of Claims 17-22, wherein the polypeptide retains at least 50% of the carbonic anhydrase activity when incubated for 1 to 3 hours at a temperature
10 of from 20°C to 50°C under high ionic strength conditions.

24. The recombinant polypeptide of Claims 1 or 2, wherein the amino acid sequence that is at least 70% identical to the amino acid sequence of SEQ ID NO: 53.

25. The recombinant polypeptide of Claim 24, wherein the polypeptide retains greater than 60% of the carbonic anhydrase activity when incubated for 5 or more hours at a pH of 8.5 to 9.5.

15 26. The recombinant polypeptide of Claims 24 or 25, wherein the polypeptide retains at least 80% of the carbonic anhydrase activity when incubated for about 24 hours at a temperature of about 25°C.

27. The recombinant polypeptide of any one of Claims 24-26, wherein the polypeptide retains at least 60% of the carbonic anhydrase activity when incubated for about 24 hours at a
20 temperature of about 40°C.

28. The recombinant polypeptide of any one of Claims 24-27, wherein the polypeptide retains at least 40% of the carbonic anhydrase activity when incubated for about 2 hours at a temperature of about 50°C.

29. The recombinant polypeptide of any one of Claims 24-28, wherein the polypeptide
25 retains at least 20% of the carbonic anhydrase activity when incubated for about 2 hours at a temperature of about 50°C.

30. The recombinant polypeptide of any one of Claims 24-29, wherein the polypeptide retains at least 50% of the carbonic anhydrase activity when incubated for 1 to 3 hours at a temperature of from 20°C to 50°C under high ionic strength conditions.

30

31. The recombinant polypeptide of Claims 1 or 2, wherein the amino acid sequence that is at least 70% identical to the amino acid sequence of SEQ ID NO: 64.

5 32. The recombinant polypeptide of Claim 31, wherein the polypeptide retains greater than 70% of the carbonic anhydrase activity when incubated for 3 or more hours, 5 or more hours, 10 or more hours, or 24 or more hours at a pH of 8.5 to 10.5.

33. The recombinant polypeptide of Claims 31 or 32, wherein the polypeptide retains at least 90% of the carbonic anhydrase activity when incubated for at least about 10 hours, or at least about 24 hours at a temperature of about 25°C.

10 34. The recombinant polypeptide of any one of Claims 31-33, wherein the polypeptide retains at least 60% of the carbonic anhydrase activity when incubated for about 24 hours at a temperature of about 40°C.

15 35. The recombinant polypeptide of any one of Claims 31-34, wherein the polypeptide retains at least 40% of the carbonic anhydrase activity when incubated for about 3 hours at a temperature of about 50°C.

36. The recombinant polypeptide of any one of Claims 31-35, wherein the polypeptide retains at least 25% of the carbonic anhydrase activity when incubated for about 3 hours at a temperature of about 50°C.

20 37. The recombinant polypeptide of any one of Claims 31-36, wherein the polypeptide retains at least 50% of the carbonic anhydrase activity when incubated for 1 to 3 hours at a temperature of from 20°C to 50°C under high ionic strength conditions.

38. The recombinant polypeptide of Claims 1 or 2, wherein the amino acid sequence that is at least 70% identical to the amino acid sequence of SEQ ID NO: 80.

25 39. The recombinant polypeptide of Claim 38, wherein the polypeptide retains greater than 70% of the carbonic anhydrase activity when incubated for 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 10 or more, 15 or more, or 24 or more hours at a pH of 8.5 to 10.5.

40. The recombinant polypeptide of Claims 38 or 39, wherein the polypeptide retains at least 60% of the carbonic anhydrase activity when incubated for about 10 hours at a temperature of about 25°C.

30

41. The recombinant polypeptide of any one of Claims 38-40, wherein the polypeptide retains at least 35% of the carbonic anhydrase activity when incubated for about 24 hours at a temperature of about 40°C.

5 42. The recombinant polypeptide of any one of Claims 38-41, wherein the polypeptide retains at least 50% of the carbonic anhydrase activity when incubated for about 1 hour at a temperature of about 50°C.

43. The recombinant polypeptide of any one of Claims 38-42, wherein the polypeptide retains at least 20% of the carbonic anhydrase activity when incubated for about 3 hour at a temperature
10 of about 50°C.

44. The recombinant polypeptide of any one of Claims 38-43, wherein the polypeptide retains at least 50% of the carbonic anhydrase activity when incubated for 1 to 3 hours at a temperature of about 20°C under high ionic strength conditions.

45. The recombinant polypeptide of Claims 1 or 2, wherein the amino acid sequence that is
15 at least 70% identical to the amino acid sequence of SEQ ID NO: 130.

46. The recombinant polypeptide of Claim 45, wherein the polypeptide retains greater than 25% of the carbonic anhydrase activity when incubated for 30 minutes, 1 hour, 2 hours, 3 hours, 5 hours, 10 hours, 24 hours, or more hours at a pH of 8.5 to 10.5.

47. The recombinant polypeptide of Claims 45 or 46, wherein the polypeptide retains at least
20 50% of the carbonic anhydrase activity when incubated for at least about 30 minutes, about 1 hour, about 2 hours, about 3 hours, about 5 hours, about 10 hours, about 15 hours, or about 24 hours at a temperature of about 25°C.

48. The recombinant polypeptide of any one of Claims 45-47, wherein the polypeptide retains at least 25% of the carbonic anhydrase activity when incubated for about 30 minutes, about 1
25 hour, about 2 hours, about 3 hours, about 4 hours, about 5 hours, about 10 hours, about 15 hours, or about 24 hours at a temperature of about 40°C.

49. The recombinant polypeptide of any one of Claims 45-48, wherein the polypeptide retains at least 25% of the carbonic anhydrase activity when incubated for about 30 minutes, about 1
hour, about 2 hours, or about 3 hours at a temperature of about 50°C.

30

50. The recombinant polypeptide of any one of Claims 45-49, wherein the polypeptide retains at least 35% of the carbonic anhydrase activity when incubated for about 1 hour, about 2 hours, or about 3 hours at a temperature of about 50°C.

5 51. The recombinant polypeptide of any one of Claims 45-50, wherein the polypeptide retains at least 50% of the carbonic anhydrase activity when incubated for at least 30 minutes, at least 1 hour, at least 2 hours, or at least 3 hours, or longer, at a temperature of from 20°C to 50°C under high ionic strength conditions.

10 52. The recombinant polypeptide of Claims 1 or 2, wherein the amino acid sequence that is at least 70% identical to the amino acid sequence of SEQ ID NO: 136.

53. The recombinant polypeptide of Claim 52, wherein the polypeptide retains greater than 25% of the carbonic anhydrase activity when incubated for at least 30 minutes, at least 1 hour, at least 2 hours, at least 3 hours, or at least 5 at a pH of 8.5 to 10.5.

15 54. The recombinant polypeptide of Claims 52 or 53, wherein the polypeptide retains at least 50% of the carbonic anhydrase activity when incubated for about 1 hour, about 2 hours, about 3 hours, about 5 hours, about 10 hours, or about 24 hours at a temperature of about 25°C.

20 55. The recombinant polypeptide of any one of Claims 52-54, wherein the polypeptide retains at least 40% of the carbonic anhydrase activity when incubated for about 30 minutes, about 1 hour, about 2 hours, about 3 hours, about 5 hours, about 10 hours, or about 24 hours at a temperature of about 40°C.

56. The recombinant polypeptide of any one of Claims 52-55, wherein the polypeptide retains at least 25% of the carbonic anhydrase activity when incubated for about 30 minutes, about 1 hour, about 2 hours, or about 3 hours at a temperature of about 50°C.

25 57. The recombinant polypeptide of any one of Claims 52-56, wherein the polypeptide retains at least 35% of the carbonic anhydrase activity when incubated for about 30 minutes, about 1 hour, about 2 hours, or about 3 hours at a temperature of about 50°C.

58. The recombinant polypeptide of any one of Claims 52-57, wherein the polypeptide retains at least 25% of the carbonic anhydrase activity when incubated for 1 to 3 hours at a temperature of from 20°C to 50°C under high ionic strength conditions.

30 59. The recombinant polypeptide of Claims 9, 16, 23, 30, 37, 44, 51 or 58, wherein the high ionic strength conditions comprise 1 M bicarbonate.

60. The recombinant polypeptide of any one of Claims 1-59, wherein the polypeptide has a melting temperature that is elevated in the presence of 1 M to 2 M bicarbonate.

61. The recombinant polypeptide of any one of Claims 2-60, wherein the polypeptide has a higher thermostability and/or melting temperature at a higher ionic strength.

5 62. The recombinant polypeptide of any one of Claims 1 or 2, wherein the polypeptide comprises an amino acid sequence that is at least 75% identical to the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 28, SEQ ID NO: 43, SEQ ID NO: 53, SEQ ID NO: 64, SEQ ID NO: 80, SEQ ID NO: 130, or SEQ ID NO: 136.

10 63. The recombinant polypeptide of any one of Claims 1 or 2, wherein the polypeptide comprises an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 3, SEQ ID NO: 28, SEQ ID NO: 43, SEQ ID NO: 53, SEQ ID NO: 64, SEQ ID NO: 80, SEQ ID NO: 130, or SEQ ID NO: 136.

15 64. The recombinant polypeptide of any one of Claims 1 or 2, wherein the polypeptide comprises an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 28, SEQ ID NO: 43, SEQ ID NO: 53, SEQ ID NO: 64, SEQ ID NO: 80, SEQ ID NO: 130, or SEQ ID NO: 136.

20 65. The recombinant polypeptide of any one of Claims 1 or 2, wherein the polypeptide comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 28, SEQ ID NO: 43, SEQ ID NO: 53, SEQ ID NO: 64, SEQ ID NO: 80, SEQ ID NO: 130, or SEQ ID NO: 136.

66. A composition comprising the recombinant polypeptide of any one of the preceding claims and a carbon dioxide-containing medium.

67. The composition of Claim 66, wherein the recombinant polypeptide is immobilized.

25 68. The composition of Claim 67, wherein the recombinant polypeptide is immobilized on a polymer, a membrane, a matrix, a micellar material, a wafer, a solid support, or a micro-particle.

69. An isolated nucleic acid encoding the recombinant polypeptide of any one of the preceding claims.

70. The isolated nucleic acid of Claim 69, wherein the polypeptide further comprises a signal peptide sequence.

30

71. The isolated nucleic acid of Claim 70, wherein the signal peptide sequence is selected from the group consisting of SEQ ID NOs: 10-15, 69-73, 81- 83, and 131-132.

72. An expression vector comprising the isolated nucleic acid of any one of Claims 69-71 in
5 operable combination with a regulatory sequence.

73. A host cell comprising the expression vector of Claim 72.

74. The host cell of Claim 73, wherein the host cell is a bacterial cell or a fungal cell.

75. A composition comprising the host cell of Claims 73 or 74 and a culture medium.

76. A method of producing a carbonic anhydrase, comprising: culturing the host cell of
10 Claim 73 or 74 in a culture medium, under suitable conditions to produce the carbonic anhydrase.

77. A composition comprising the carbonic anhydrase produced in accordance with the method of Claim 76 in supernatant of the culture medium.

78. A method for extracting carbon dioxide from a carbon dioxide-containing medium, comprising: contacting the carbon dioxide-containing medium with the polypeptide of any one of Claims
15 1-64 to yield a medium reduced in carbon dioxide.

79. The method of Claim 78, wherein the carbon dioxide-containing medium is selected from the group consisting of a gas, a liquid, and a multi-phase mixture.

80. The method of Claim 78, wherein the carbon dioxide-containing medium is a gas selected from the group consisting of a flue gas, a raw natural gas, a syngas, a hydrogen gas, and a
20 biogas.

81. The method of Claim 78, wherein the carbon dioxide-containing medium is a by-product of combustion or fermentation.

82. A method for regulating pH of a medium containing one or both of CO₂ and bicarbonate, comprising: contacting the medium with the polypeptide of any one of Claims 1-65 to yield a medium
25 with an altered pH.

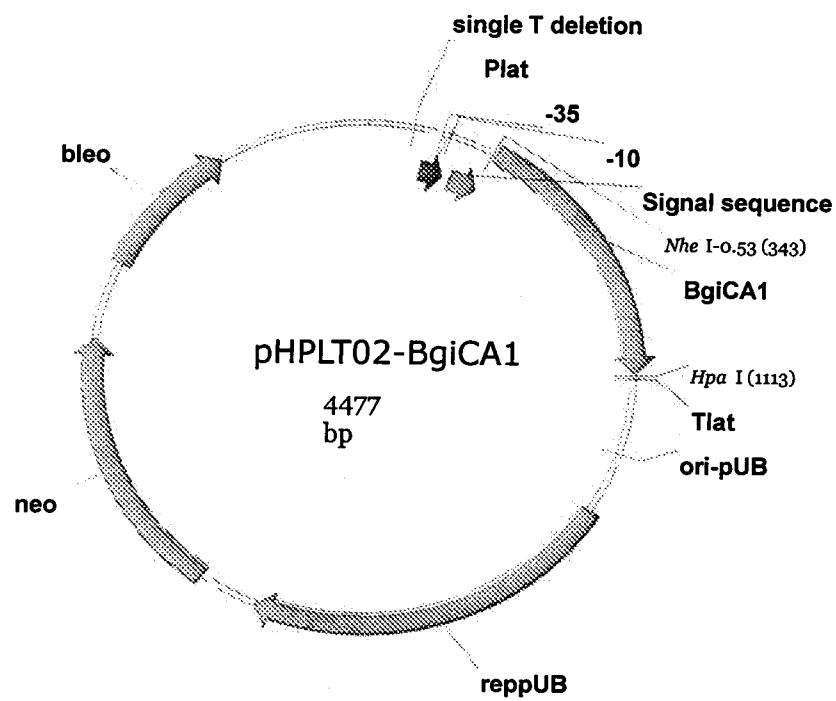


Figure 1

Figure 2A

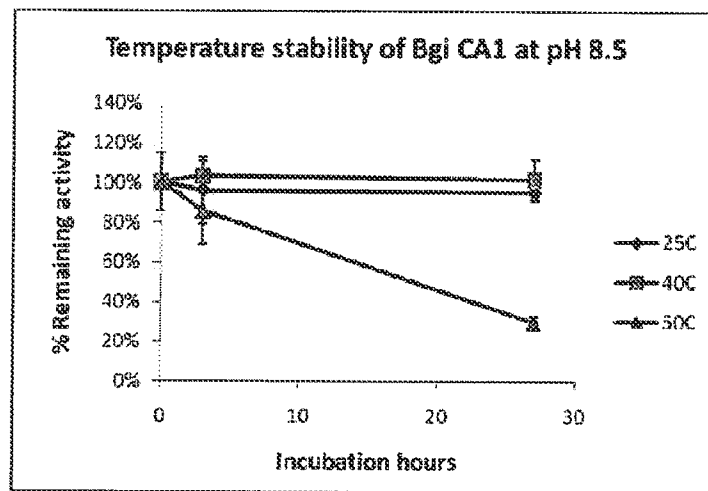


Figure 2B

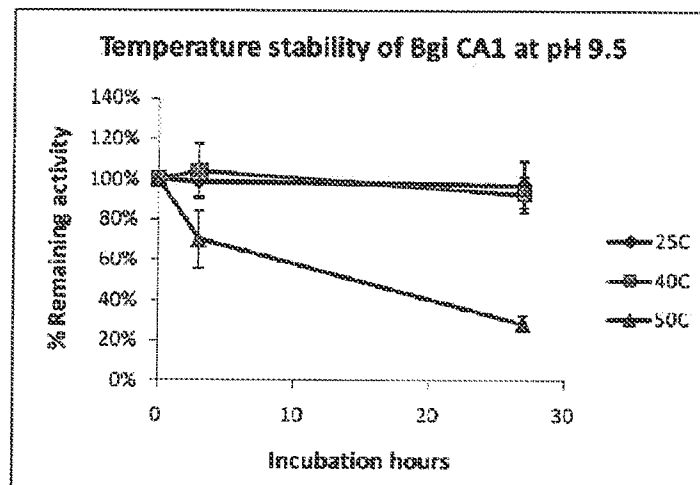
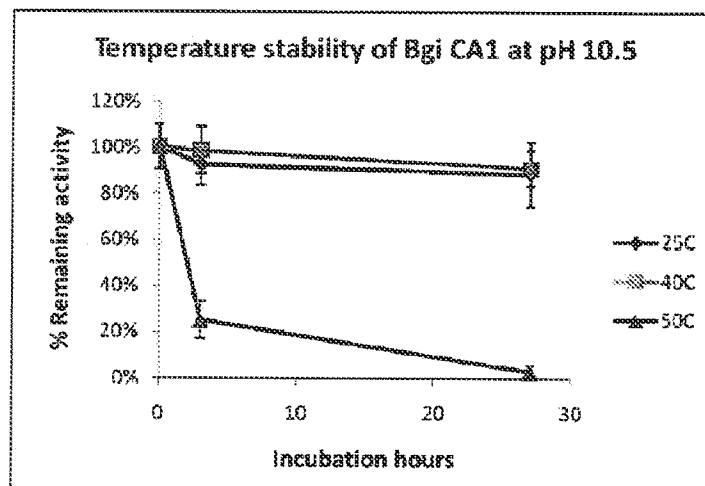
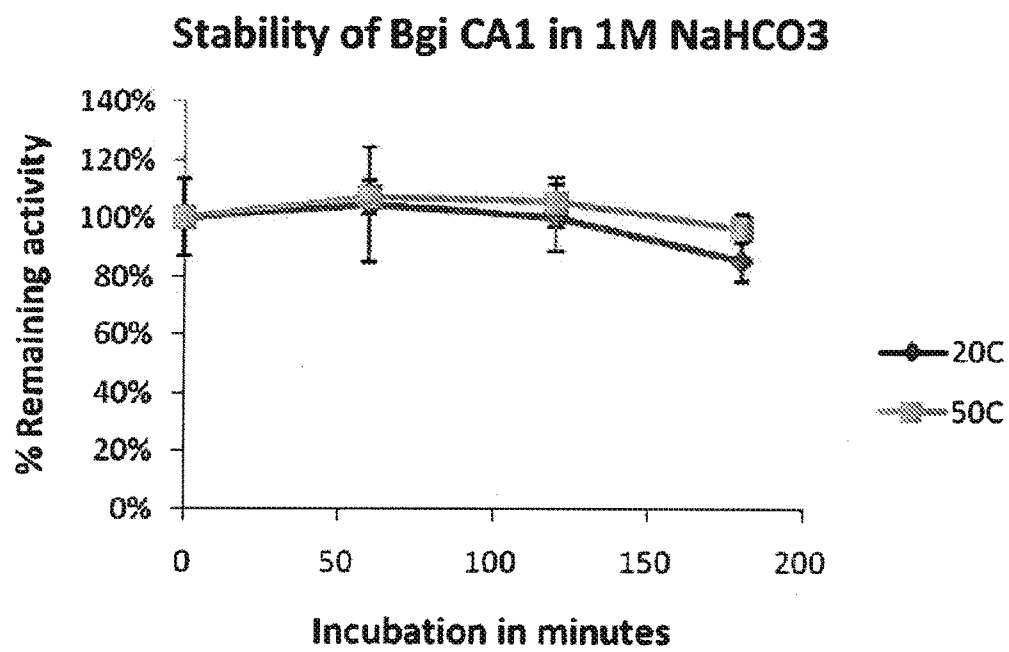


Figure 2C



**Figure 3**

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YP_176677_Bacillus_clausii      -----MKRSHLFTSITLASVVTLATAPASAA
SgiCA1                          -----ANGNHVSSSLINS
YP_003868697_Paenibacillus_polymyxa -----MKKNWKGCLFSIFAILLVSVTGONSQPAISSTPSTSSSSA
YP_003944556_Paenibacillus_polymyxa -----MKKHWRGCLFSIFSIIALSVTGCTPSATSSSTTSATSSSSA
ZP_04209878_Bacillus_cereus      -----MGCNTAKQE
ZP_04248491_Bacillus_cereus      -----MKGLKFFVYKTPILITMSVVLMSGCNTAKQE
YP_003428428_Bacillus_pseudofirmus -----MKRKSIIYLLVAASLALAACAETETAPVETFPITDSTEFSD
NP_241226_Bacillus_halodurans     -----MKKYLWGKTCCLVVSLSVMVTACSSAPSTPEVDEPSET
NP_946147_Rhodopseudomonas_palustris -----MDRRNIIKAFAGLALCPLC
YP_488219_Rhodopseudomonas_palustris -----MEPILIPFHRPMDRRAILKAFAGLALCPLC
NP_767777_Bradyrhizobium_japonicum MGRKDRVGEPIINSTRDGHCDSPRLSPRLRAHRRSNSSWGNMNRHALKALAGLALCPVC

YP_176677_Bacillus_clausii      SFLSPLQALKASWSYEGETGPEFWGDLDEAFACSNKGESPINLFYDR---EQTSKWNWA
SgiCA1                          PYDRLTANASHDWSYSGPTGPEFWGELDSEFKACSNGTQQSPIALDPTD---VGDEKWSLD
YP_003868697_Paenibacillus_polymyxa TNAHAAVQKGFPHWSYEGDQGFPHWGELEKDFVACGNGHEQS FVNIETHLEASQTRQPLQ
YP_003944556_Paenibacillus_polymyxa NNVHAVVQKSPHWSYEGDEGPEHWGELEKDFVACGNGQEQSPINIEHSHLEASHTQQLQ
ZP_04209878_Bacillus_cereus      APHKQSTKENTQWSYEGTTGPEHWGELKPEYKICLNGQEQSPIDIKTEQIKSTVDNNLLQ
ZP_04248491_Bacillus_cereus      APQKQSTKENTQWSYKSTGPEHWGELKPEYNMCLNGQEQSPIDIKTEQIKSTVDNNLLQ
YP_003428428_Bacillus_pseudofirmus ETQEESTBELTEWSYEGESGPEHWGHLHASYSACVOGSEQS PINIDLAENANQIQEIID
NP_241226_Bacillus_halodurans     HEETSOGAHEVWWSYTGDTGPEHWAEILDSEYGACAGGEEQSPINLDKAE---AVDTDTETQ
NP_946147_Rhodopseudomonas_palustris ATAGRAAEGANHWGYEGEGGPAKWGEIDPANQICSIGVQQSPVDIRSTV----SANLFPLQ
YP_488219_Rhodopseudomonas_palustris ASAGVAGEGGHWWGYEGDGGPAKWGEIDPANQFCSVGVQQSPIDIGTTI----GANLYPIE
NP_767777_Bradyrhizobium_japonicum KPAFAAE---GVHWSYEGAGAPAKWGDLDAAKACAVGLQQSPIDIEATI---KSQLP TLK

          *. * . * * . . . * * * : * * : * * :
YP_176677_Bacillus_clausii      FSYSEAAFSVENNGHTIQANV---ENEDAGGLEINGEAYQLIQFHFTPSEHTIEETSFP
SgiCA1                          LDYAKTEFSIENNGHTIQANVVEKKQGPSNQLTLGDSTYELVQFHFHSPSEHTLAGESYE
YP_003868697_Paenibacillus_polymyxa VHYTNTKASILNNGHTVQINV----ADASNIMLDGTRFTLKQFHFHHPSEHQIDGKNAE
YP_003944556_Paenibacillus_polymyxa VHYSTTKVSI LNNGHTVQVNA----ASPSNDIVVDGTRFTLKQFHFHHPSEHQIDGKNAE
ZP_04209878_Bacillus_cereus      INYQPIFSIKNNCHSIEGKA----NSSDNYLTLCENRYTLNQFHFTPSEHQFECKHAD
ZP_04248491_Bacillus_cereus      INYQPIFSIKNNCHSIEGKA----NSSDOYLTLCENRYTLNQFHFTPSEHQFECKHAD
YP_003428428_Bacillus_pseudofirmus IQYEPASFSILVNNGHTIQKNA----VDENNAITLDGQEQYQLVQFHFHTPSEHQFNGEHYD
NP_241226_Bacillus_halodurans     VHYEPASFTIKNNGHTIQAEI----TSDGNTIEIDGKEYTLVQFHFHHPSEHEMEGKNLD
NP_946147_Rhodopseudomonas_palustris VQWADTADTIINNGHTIQNLV-----AEGSTLKLGGATFKLVQFHFHHPSEHQIDGKSPF
YP_488219_Rhodopseudomonas_palustris IRWADTADTIIVNNGHTIQNLV-----AEGSHLKLGGVTFKLVQFHFHHPSEHLIDGKNFP
NP_767777_Bradyrhizobium_japonicum LNWGKSADTIIVNNGHTIQNLV-----AEGSTLTLGGVVKYKLLQVHFHHPSEHMIGGKNFP

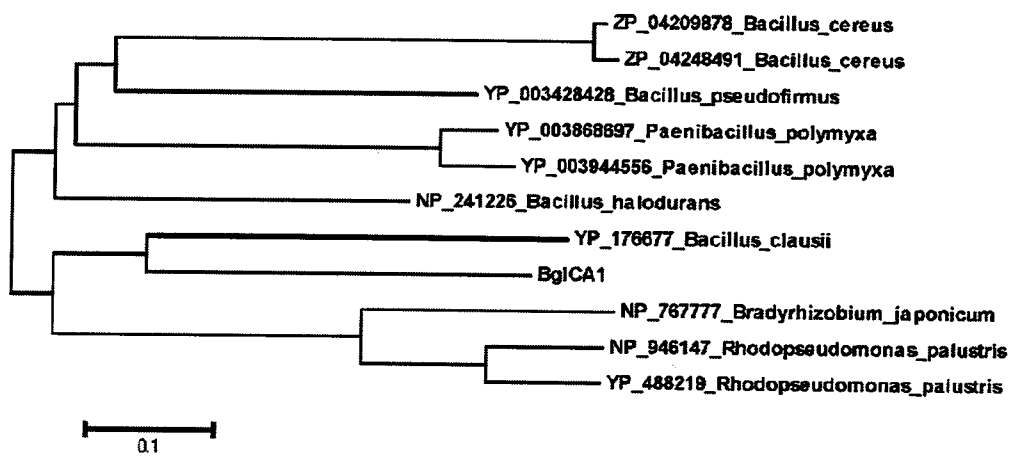
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```

Figure 4

YP_176677_Bacillus_clausii	MELHLVHANHAGOLAVLGVLMEMGNDHEGIEAVWEVMPPEEG---TAAYSISLDPNLFLPE
BgiCA1	MEVHLVHKDEQDNLAVLGVLMEEGEKKNKALKDMWKKMPTSVG---TSTKTIKLNPSSELVPT
YP_003868697_Paenibacillus_polymyxa	MELHFEVHQSDNGSTAVLGVLIQSGKENKAFNRIWSKLPKDNSQEAALDKEINLAALLPK
YP_003944556_Paenibacillus_polymyxa	MELHFEVHQSDTGSTAVLGVLIQSGKENKAFNRIWSKLPKDISQEA--LDDEVNLAALLPK
ZP_04209878_Bacillus_cereus	MELHLVQNDDQGLVVGIMIKEGQKNEGFAAMWQNLPRKKN- IKADVQHTIDIKQILPS
ZP_04248491_Bacillus_cereus	MELHLVQNDDQGLAVVGIMIKEGQKNEGFAAMWQNLPRKKN- IKADVQHTIDIKQILPS
YP_003428428_Bacillus_pseudofirmus	MELHLVHQDINGNLAVLGVLMIEGAENEELAPAWGELPEEETENEVALEEPINLQNLDP
NP_241226_Bacillus_halodurans	MELHFEVHKNEDELAVLGVLMKAGEENEELAKLWSKLPAAETEENISLDESIDNALLPE
NP_946147_Rhodopseudomonas_palustris	MEVHFEVHMDSGTLGVVGVLHQEGAANAFAKIVATMPQSEG-PAVKADAGINPNALLPA
YP_488219_Rhodopseudomonas_palustris	MEVHFEVHRADSGTLGVVGVLHQPGKANAAFSKIVATMPQSEG-PAVKADPAIDPNALLPE
NP_767777_Bradyrhizobium_japonicum	MEAHFEVHRNDAGGLAVVGVLMAEGKFNPAFGKIVKTMFAADG-PAVKADASIDPLAMLET
	** *:*** . *:*** * : : : *
	::*
YP_176677_Bacillus_clausii	SVTAYQYDGSLLTPPCSEGKWTVLNDTISISSETQLDAFRDIYFQNYRFVQELGDREIGF
BgiCA1	DLSTFYQYDGSLLTPPCSEGKWSVSDSSITLSSEQLQAFQOLYPNNYRPIQDLGDREVG
YP_003868697_Paenibacillus_polymyxa	DLHSVRYNGSLTTPPCTEHVNWTVLEQPIEMSADQINQFAAIFPDNHRFPVQQLGTRELT
YP_003944556_Paenibacillus_polymyxa	DLHSVRYNGSLTTPPCTEHVNWTVLEQPIEMSADQIKQFAAIFPDNHRFPVQQLGTRELK
ZP_04209878_Bacillus_cereus	DYSSFRYMGSLTTPPCTENVQWIVMRQTIEMSKKQIKVFHKLFPNTNRFPVQPIINGRAVL-
ZP_04248491_Bacillus_cereus	DHSSEFRYMGSLTTPPCTENVQWIVMRQTIEMSKKQIKVFHKLFPNTNRFPVQPIINGRAVL-
YP_003428428_Bacillus_pseudofirmus	DQSSEFRYNGSLTTPPCTEEVKWIVFKEPIQKSABQIQAFQEIYEENHRFPVQPLNERG----
NP_241226_Bacillus_halodurans	SKGEGFHYNGSLTTPPCSEGKWTVLSEFIVTSQEQIDAFAEIIFPDNHRFPVQPNDRDQVD
NP_946147_Rhodopseudomonas_palustris	KLGYVRYEGSLTTPCSEVVDWMVLTDPIITVAEDVAFAKLYPMNARFPVQKDNRRFVLQ
YP_488219_Rhodopseudomonas_palustris	TRNYVRYEGSLTTPCSEIVDWMVLASPIITVAADDIAFAKLYPMNARFPVQKDNRRFVLQ
NP_767777_Bradyrhizobium_japonicum	RLSYFRYPGSLTTPCSEVVENLLLTPIQVSAADVAFAKLYPMNARFPVQKDNRRYVLR
	: * *****: * *. * : . * : : : * : : * ** : . *
YP_176677_Bacillus_clausii	HYH-
BgiCA1	HY--
YP_003868697_Paenibacillus_polymyxa	DK--
YP_003944556_Paenibacillus_polymyxa	DK--
ZP_04209878_Bacillus_cereus	----
ZP_04248491_Bacillus_cereus	----
YP_003428428_Bacillus_pseudofirmus	----
NP_241226_Bacillus_halodurans	VITE
NP_946147_Rhodopseudomonas_palustris	SN--
YP_488219_Rhodopseudomonas_palustris	SN--
NP_767777_Bradyrhizobium_japonicum	ST--

Figure 4 Continued

**Figure 5**

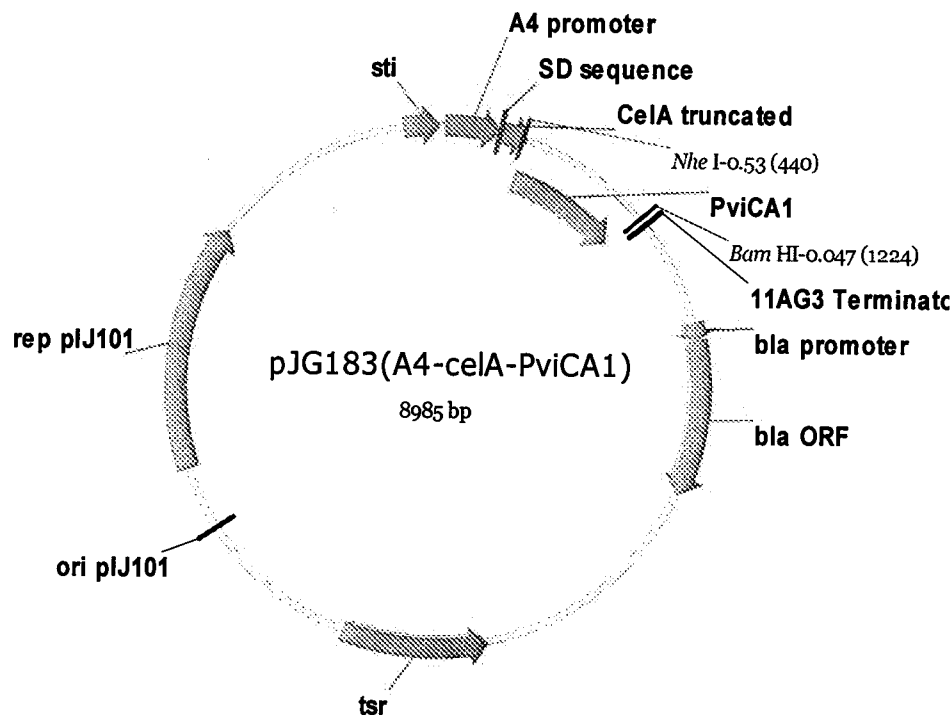


Figure 6

Figure 7A

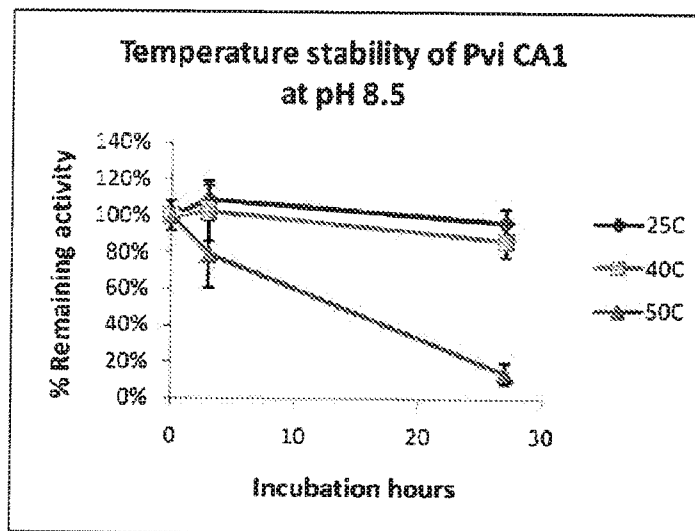


Figure 7B

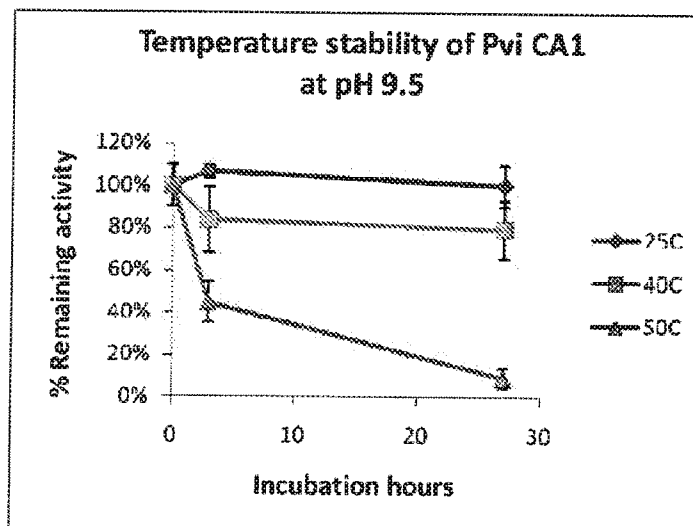
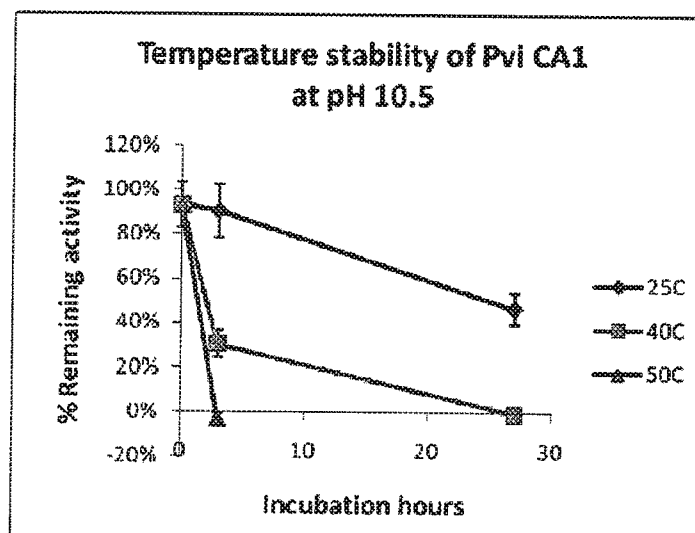
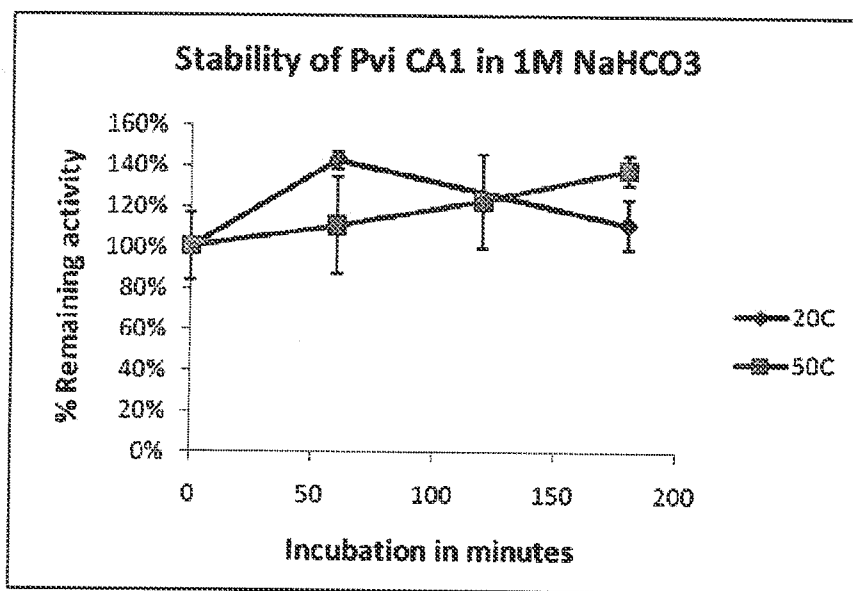


Figure 7C



**Figure 8**

YP_003868697_Paenibacillus_polymyxa	-----MKKNWKGCLFSIFAILLVSVTCNSEQPAISS
YP_003944556_Paenibacillus_polymyxa	-----MKKHWRGCLFSIFSIIALLSVTGCTPSATSSST
NP_241226_Bacillus_halodurans	-----MKKYLWGKTCLVVSLVMVTACSSAPST
YP_003166850_Candidatus_accumulibacter	-----MIVTTTDTGTFSMHFEVIRLSSAFVLVLSLLP
YP_287120_Dechloromonas_aromatica	-----MRHLIIASLLAALPWA
ZP_05029585_Microcoleus_chthonoplastes	-----MKNSTFTFIVFVLLA
YP_419944_Magnetospirillum_magnetotacticum	-----MDRRSFLKGATGAACLCG
ZP_00056365_Magnetospirillum_magnetotacticum	-----
YP_003610599_Enterobacter_cloacae	-----MKHILTA
ZP_08017465_Lautropia_mirabilis	MINIPNLRKAIMHVRQLLVPLLSGLFLSSAAMAETAQPAADAAGASPGQ
PvICA1	-----DG
YP_003868697_Paenibacillus_polymyxa	TPSTSSSSATNAH-----
YP_003944556_Paenibacillus_polymyxa	TSATSSSSANNVH-----
NP_241226_Bacillus_halodurans	EPVDEPSETHEET-----
YP_003166850_Candidatus_accumulibacter	PGVGAAWQVISAEPGKRVEIDRASIRFDETGKSVAQGRIVLEKPIVDPKT
YP_287120_Dechloromonas_aromatica	ASAAPTWQTISSEPGKRIEIDRTSLKR-EGSTVQAQGRIVLEKELTDKAS
ZP_05029585_Microcoleus_chthonoplastes	SCLIIAPNVKSS-----
YP_419944_Magnetospirillum_magnetotacticum	TCGGSIAEAVAAE-----
ZP_00056365_Magnetospirillum_magnetotacticum	-----MASE-----
YP_003610599_Enterobacter_cloacae	SLFALSASVFAGS-----
ZP_08017465_Lautropia_mirabilis	VPAASSDSKPADK-----
PvICA1	ETSAPPPPATD-----
YP_003868697_Paenibacillus_polymyxa	-----
YP_003944556_Paenibacillus_polymyxa	-----
NP_241226_Bacillus_halodurans	-----
YP_003166850_Candidatus_accumulibacter	SSSYRIVEAVNRYDCASRSYSTLKRSYFKDEGDLREEEVKVQIEMPVRT
YP_287120_Dechloromonas_aromatica	GAGYRVIEAITHYDCNTRNANTIKRIFFKKNEVIREEEIKGS-DLPVRT
ZP_05029585_Microcoleus_chthonoplastes	-----
YP_419944_Magnetospirillum_magnetotacticum	-----
ZP_00056365_Magnetospirillum_magnetotacticum	-----
YP_003610599_Enterobacter_cloacae	-----
ZP_08017465_Lautropia_mirabilis	-----
PvICA1	-----

Figure 9

YP_003868697_Paenibacillus_polymyxa	-----
YP_003944556_Paenibacillus_polymyxa	-----
NP_241226_Bacillus_halodurans	-----
YP_003166850_Candidatus_accumulibacter	GMLDDKLLREVCRRPKPGPDAATAASKVADKVNQAAGELRKANEALVQKEV
YP_287120_Dechloromonas_aromatica	GTLDOKVLREVCRRPKESPA-----ELAKKANEAGELKAANDALLKKEM
ZP_05029585_Microcoleus_chthonoplastes	-----
YP_419944_Magnetospirillum_magnetotacticum	-----
ZP_00056365_Magnetospirillum_magnetotacticum	-----
YP_003610599_Enterobacter_cloacae	-----
ZP_08017465_Lautropia_mirabilis	-----
PviCA1	-----
YP_003868697_Paenibacillus_polymyxa	-----
YP_003944556_Paenibacillus_polymyxa	-----
NP_241226_Bacillus_halodurans	-----
YP_003166850_Candidatus_accumulibacter	KRANLQTPNVDKAEAEPRSSPAAKPTAAAAMPARVHSARPAAPRGE---
YP_287120_Dechloromonas_aromatica	AKAEKPATIKASDVFDKEAENGAIPSIRPNLKAATESAKETAPAPTAAA
ZP_05029585_Microcoleus_chthonoplastes	-----
YP_419944_Magnetospirillum_magnetotacticum	-----
ZP_00056365_Magnetospirillum_magnetotacticum	-----
YP_003610599_Enterobacter_cloacae	-----
ZP_08017465_Lautropia_mirabilis	-----
PviCA1	-----
YP_003868697_Paenibacillus_polymyxa	-----AAVQKGF
YP_003944556_Paenibacillus_polymyxa	-----AVVQKSP
NP_241226_Bacillus_halodurans	-----SCGAHEV
YP_003166850_Candidatus_accumulibacter	-----TSPAIAHANG
YP_287120_Dechloromonas_aromatica	PAKAVAPAKAATVVVHTTAPAPKARKPARSEGYLELTHSEPAQHAQI
ZP_05029585_Microcoleus_chthonoplastes	-----NTHSV
YP_419944_Magnetospirillum_magnetotacticum	-----CA
ZP_00056365_Magnetospirillum_magnetotacticum	-----CA
YP_003610599_Enterobacter_cloacae	-----AP
ZP_08017465_Lautropia_mirabilis	-----PA
PviCA1	-----EV

Figure 9 Continued

YP_003868697_Paenibacillus_polymyxa
 YP_003944556_Paenibacillus_polymyxa
 NP_241226_Bacillus_halodurans
 YP_003166850_Candidatus_accumulibacter
 YP_287120_Dechloromonas_aromatica
 ZP_05029585_Microcoleus_chthonoplastes
 YP_419944_Magnetospirillum_magnetotacticum
 ZP_00056365_Magnetospirillum_magnetotacticum
 YP_003610599_Enterobacter_cloacae
 ZP_08017465_Lautropia_mirabilis
 PviCA1

 YP_003868697_Paenibacillus_polymyxa
 YP_003944556_Paenibacillus_polymyxa
 NP_241226_Bacillus_halodurans
 YP_003166850_Candidatus_accumulibacter
 YP_287120_Dechloromonas_aromatica
 ZP_05029585_Microcoleus_chthonoplastes
 YP_419944_Magnetospirillum_magnetotacticum
 ZP_00056365_Magnetospirillum_magnetotacticum
 YP_003610599_Enterobacter_cloacae
 ZP_08017465_Lautropia_mirabilis
 PviCA1

 YP_003868697_Paenibacillus_polymyxa
 YP_003944556_Paenibacillus_polymyxa
 NP_241226_Bacillus_halodurans
 YP_003166850_Candidatus_accumulibacter
 YP_287120_Dechloromonas_aromatica
 ZP_05029585_Microcoleus_chthonoplastes
 YP_419944_Magnetospirillum_magnetotacticum
 ZP_00056365_Magnetospirillum_magnetotacticum
 YP_003610599_Enterobacter_cloacae
 ZP_08017465_Lautropia_mirabilis
 PviCA1

HWSYEGDQGFHWELEKDFVACNGHEQSFVNIETHLEASQTRQPLQV
 HWSYEGDEGPFHWELEKDFVACNGQEQSFNIEHSHLEASHTQQLQV
 HWSYTGDTGPERWAELOSEYACAGQEEQSPINLDKA---EAVDTDTETQV
 HWAYEGEGGPDNWKGLKPEYATCATGKRQSPIDIR---DGFRVLEPIQF
 HWAYOGAGAPENWPNLDPKNKVCAGERQSPIDIK---DGKVDLEPIKF
 DWGYSGDESPEKWDLSPEFETCKLGKTQSPIDLN---DMSASSAGSLEF
 HWAYEGHGGPKWGMLSPEYAACSMGREQSPVOLT---RPIAAIIGDFMA
 HWAYEGHGGPKWGMLSPEYAACSMGREQSPVOLS---KPIAAIIGDFLT
 HWYEGKSGPENWGLSDEFATCKTKGKFSQSPIDIR---NAYNATLPPLEM
 HWSYGGSEGPAYWGLSADYSQCSIGRNQSFVDLNQD--DAIRSNKDAVQV
 HWSYEGDTGPDNWWGLSDEFVECSIGEAQSPVLPDHN---ADETTTEPPTV

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 HYTNKASILNNGHTVQINVDASNNIMLDGTRFTLKQFHFHHPSEHQID
 HYSTTKVSIILNNGHTVQVNAASPSNDIVVDGKFTLKQFHFHHPSEHQID
 HYEPSAFTIKNNGHTIQAETTSQDNTIEIDGNEYTLVQFHFHHPSEHEME
 VYRPSQFRVVDNGHTVQVEV--SGSSISLLG-RSYDLTQFHFHHPSEERVN
 KYQPSTFRIVDNGHTVQVEV--GDSSISLTG-KTYELVQFHFHHPSEERVN
 TYKYTPYKVINNGHAEVAYKAGSSIKIEG-KRYELLQFHFHHPSEHTIK
 AWRFPVPLRVQNNGHTIQVDCSGGGTILMDG--KSYDLLQFHFHHPSEHTVD
 AWRFPVPLRVQNNGHTIQVDCAGGGSLMDG--KSYDLLQFHFHHPSEHTVD
 NFHTAAEKLVNNGHTLQVT--ASDEDEFRLODQIFTLRQYHFHTPSENIN
 DYQPMGYELVNNGHTLQATPAGSQPFLQIGSRTFTLKQFHFHHPSEHTFK
 TTWPTVGESVDTGHTIQLVPDGDASEVEWQDFTFDLAQVHFHHPSEHTIE

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 GKNAEMELHFVHQSDNGSTAVLGVLIQSGKENKAFNRISKLPRKNSQEA
 GKNAEMELHFVHQSDTGSTAVLGVLIQSGKENKAFNRISKLPRKNSQEA
 GKNLDMELHFVHKNEGELAVLGVLKAGEENEELAKLWSKLPARETEEN
 GKAFDMVAHLVHRAEDGRIAVVAVLLEKGLNFVQSVWNNLPLEKNEYV
 GQRFDMMVHLVHKSDDGQLAVVAVLLEKGTENPFITLWNNMPLKNNMAV
 GSDYPMEANLHVHKSQDQGLAVIGVFLNEGQYNPFITLWNNMPLKNNMAV
 GAFFDMECHFVHKAADGGLAVLGVMIAKGAANPALEAIWQVMPAKAGEAA
 GAFFDMECHFVHKAADGGLAVLGVMIAKGAANPALEAIWQVMPAKAGETA
 GNSFPLEAHFVHKAKEGDVAVLAVMFEVGPENSALNPELLARLPKEKDHEI
 GRKFPLEHLVHGAEDGALAVLAVVQDEGENPALAPLVAE--SLSKGQTR
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Figure 9 Continued

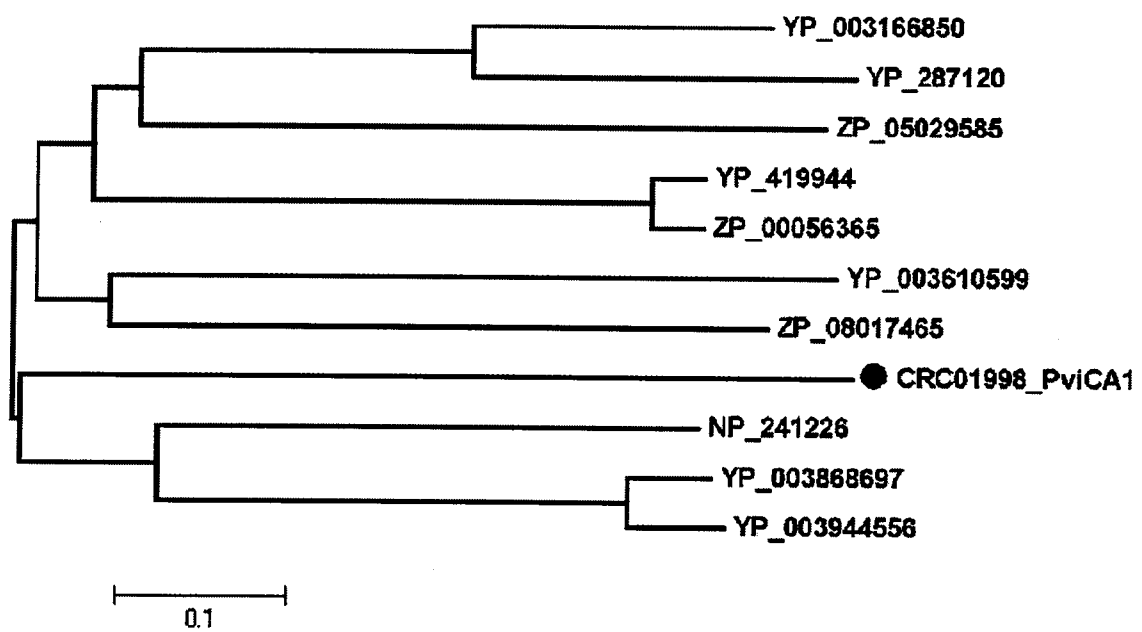
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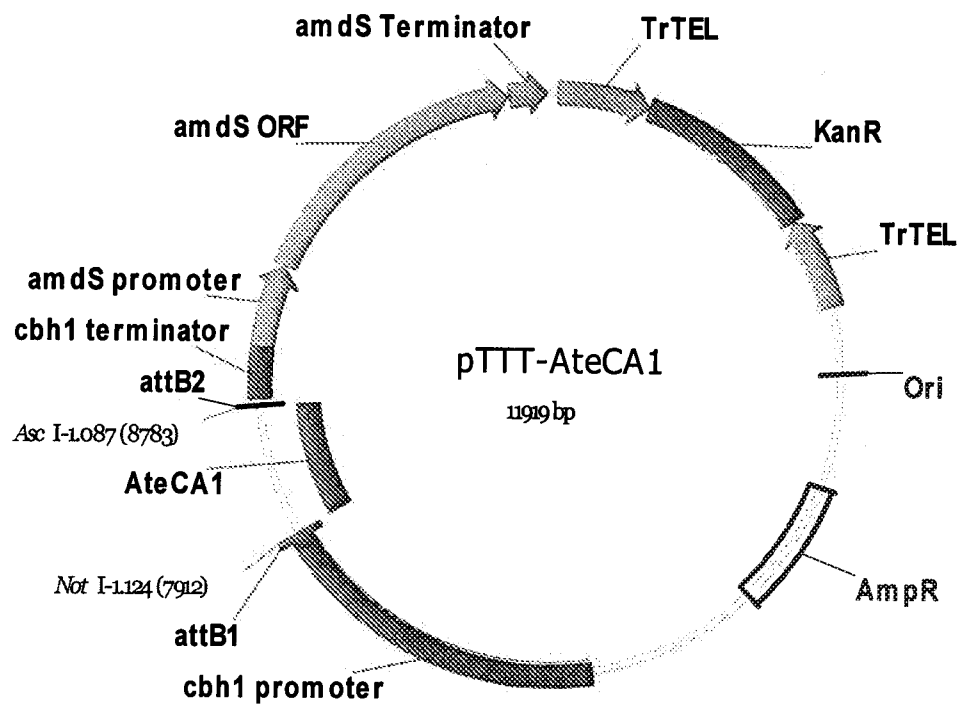
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YP_003944556_Paenibacillus_polymyxa      V-LDEDVNLAAALLPKDLHSVRYNGSLTTPPCTEHVNWTVLEQPIEMSAHQ
NP_241226_Bacillus_halodurans             ISLDESIDLNALLPESKEGFHYNGSLTTPPCSEGVKNTVLSEPIVTSQEQ
YP_003166850_Candidatus_accumulibacter    TFPELSIDVSQQLLPQOHSYTYMGSLTTPPCSEGVNLWLVRQPQIQSPEQ
YP_287120_Dechloromonas_aromatica        APFTTTIOLNLTLLPATRNYYTYMGSLTTPPCSEGVNLWLVRQPQVVSQDQ
ZP_05029585_Microcoleus_chthonoplastes   VRG-VTVNASALPPKDKSFYHYTGSLTTPPCTEGVNWVYVLRQPIETSSQ
YP_419944_Magnetospirillum_magnetotacticum TAT-SMLDASMLLEKDPVTFRYAGSLTTPPCTEVVQWVVRQAITASAEQ
ZP_00056365_Magnetospirillum_magnetotacticum TGT-SMLDASMLLEKDPVTFRYAGSLTTPPCEVVQWVVRQAVTASAEQ
YP_003610599_Enterobacter_cloacae        SIO-KHLDLRPLFPADLHYRFSGSLTTPPCTEGLRWLVKNTVTLSEKQ
ZP_08017465_Lautropia_mirabilis          KLA-EPLDIRPLLEKLSYFRINGSLTTPPCSEGVTVVVFSSPVKASKAQ
PviCA1                                     LPL-----DVAAMLPTDPTFEENTGSLTTPPCTEGVNWVYVHEPIELSAEQ
                                           :   : *          ***** : * * .. * *

YP_003868697_Paenibacillus_polymyxa      INQFAAIFPD-NHRPVQQLGTRELTADK-----
YP_003944556_Paenibacillus_polymyxa      IKQFAAIFPD-NHRPVQQLGTRELKADK-----
NP_241226_Bacillus_halodurans             IDAFASIFPD-NHRPVQPNDRDQVYDVITE-----
YP_003166850_Candidatus_accumulibacter    LAIFSRLYRN-NARPVQPNFARMIKESR-----
YP_287120_Dechloromonas_aromatica        INIFSRLYKN-NARPIQPSGGALIKEGR-----
ZP_05029585_Microcoleus_chthonoplastes   LAKFQSVYSG-NARPVQPLNKRIVINTKEM-----
YP_419944_Magnetospirillum_magnetotacticum LAAPAKLFPP-NARPVQPLNRRKLLLOVM-----
ZP_00056365_Magnetospirillum_magnetotacticum LAAPAKLFPP-NARPVQPLNRRKLLLOVM-----
YP_003610599_Enterobacter_cloacae        LEMFKQALEHSNNRPLQPLNGRVIVQ-----
ZP_08017465_Lautropia_mirabilis          IEALGQIMGGPNNRNPVQPLNARIIVDDDR-----
PviCA1                                     IAVLRDAYDN-TARPTQLLGDVVYEGTIOVEAEAAH
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Figure 9 Continued

**Figure 10**

**Figure 11**

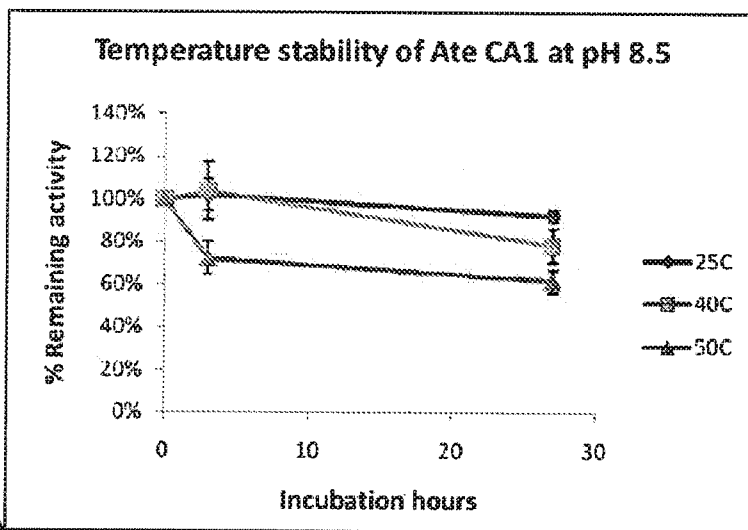


Figure 12A

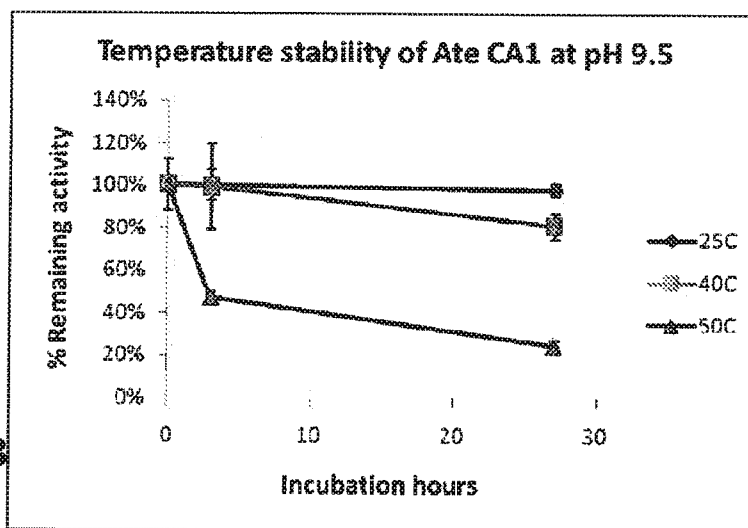


Figure 12B

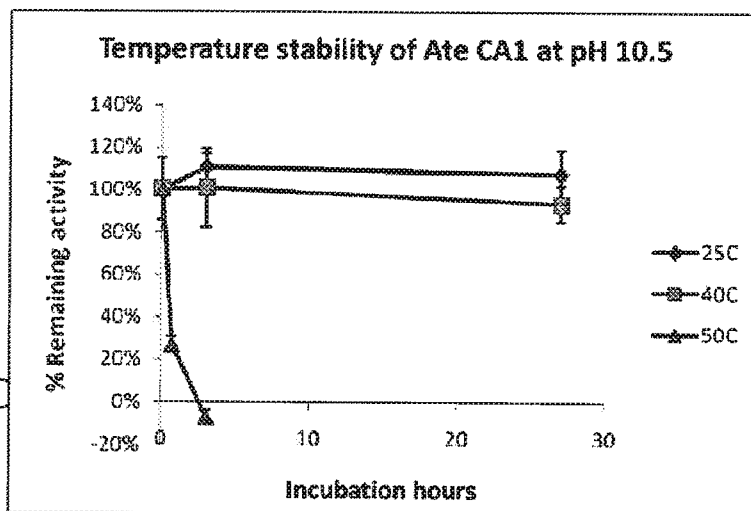
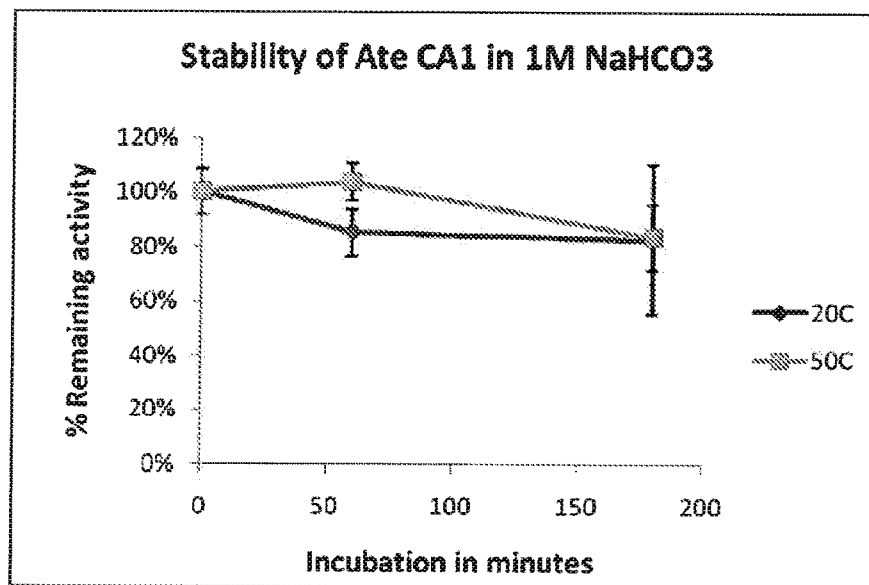


Figure 12C

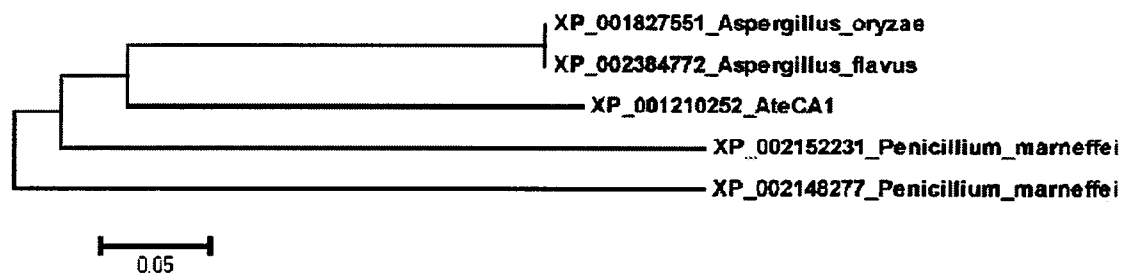
**Figure 13**

XP_001827551_Aspergillus oryza	-----MKFATLLPLLAGASAFCIHSPVMRRAAGGLDD
XP_002384772_Aspergillus flavus	MWLPCFQPIIFFRCSSVMKFATLLPLLAGASAFCIHSPVMRRAAGGLDD
XP_001210252_AteCA1	-----MKLTAAVLSLAVAASASCIRHARRADGVVE
XP_002152231_Penicillium_marneffei	-----MDFALAFVSLITAASASCIYGTSLMPRAAEGVVD
XP_002148277_Penicillium_marneffei	-----MMKLSSLLFFALPAAAAHIHARAAGHVID
	* * * : :
XP_001827551_Aspergillus oryza	ANKFNYTGLGGPLNWWYGLD-EANEACAKGKHQSPIVIDSAADYAAAGSL
XP_002384772_Aspergillus flavus	ANKFNYTGLGGPLNWWYGLD-EANEACAKGKHQSPIVIDSAADYAAAGSL
XP_001210252_AteCA1	TNSYNYTEMGGPLNWWYGLDPEANSACATGKHQSPIVIHSEDIDVVSFGL
XP_002152231_Penicillium_marneffei	ILSFNYTATGGPLNWWLLNTTANNACATGKNQSPVDIVMEGITYAIPGSV
XP_002148277_Penicillium_marneffei	TNPFNYTGLGGPLNWWYGLNKTANEACAKGMRQSPINIDTHTIEYAATGSV
	:*** *****: *: **.*.*.* ,***; * * * . **:
XP_001827551_Aspergillus oryza	KLDLPLADGSKLENLGFGLQVTLTNGSLTANSKTYTLAQFHFHTPSEHHV
XP_002384772_Aspergillus flavus	KLDLPLADGSKLENLGFGLQVTLTNGSLTANSKTYTLAQFHFHTPSEHHV
XP_001210252_AteCA1	KFDIPKADYAKFENLGSGLEVLTNGSLTVGNKSLPLAQFHFHTPSEHRV
XP_002152231_Penicillium_marneffei	KLDIPCAGGVELENLNNVVEVVANNGLTTPESIYKLAQFHFHTPSEHRV
XP_002148277_Penicillium_marneffei	NFNISNVASAKFENSGFGLVLTNGSLVNNITYYLDNFHFHTPSEHRV
	::::: . :;* . :;* .**:*.. * :*****;*

Figure 14

XP_001827551_Aspergillus oryza	NEEHFPMEVHFVFQTAAKETAVVGFFFQLSEVGDSVPLFDSVFAPIDNIP
XP_002384772_Aspergillus flavus	NEEHFPMEVHFVFQTAAKETAVVGFFFQLSEVGDSVPLFDSVFAPIDNIP
XP_001210252_AteCA1	NDEYYPMEVHFVFQNKAKDTAVVGFFFQLSELGYSVPLFDITFDHVLEIE
XP_002152231_Penicillium marneffei	NEEYFPMEAHFVFENEASQIAVAALFQLSESGASNPLFDSVFAHLDIT
XP_002148277_Penicillium marneffei	DEEYFPMECHFGFVSDDYKIAVVGFFMEISRFGYTTPLLDVFAHLDIT
	::*::*** ** * . . **..*:::*. * : **::** : :*
XP_001827551_Aspergillus oryza	DAGTSTTTGQLDFGGLLDHFNHGVYQYTGSLTTPPCTEEVMWNLSTEPL
XP_002384772_Aspergillus flavus	DAGTSTTTGQLDFGGLLDHFNHGVYQYTGSLTTPPCTEEVMWNLSTEPL
XP_001210252_AteCA1	EPGAFTHTGEMDFAGLTHHLYMHGIYQYSGSLTTPPCSEDVAWYLSTEPL
XP_002152231_Penicillium marneffei	TPGTFTKTGPLDFTTVNQHFNSNHGIFQYSGSLTTPPCSEGLSWYISTEPM
XP_002148277_Penicillium marneffei	KPGTFTKTGPLDFSGLSHFNKYGVYTYSGSLTTPPCTEGVSWFISTEPL
	.*: * ** :** : *: :*: *:*****:* : * :****:
XP_001827551_Aspergillus oryza	PLTVQGYNKVKKIIKYNARYTQNALGQDNLLEVAQAQKLSIR-----
XP_002384772_Aspergillus flavus	PLTVQGYNKVKKIIKYNARYTQNALGQDNLLEVAQAQKLSIR-----
XP_001210252_AteCA1	PLTVQDYNKVKKVLKYNARYTQNALGEDNLLEVAAKSLN-----
XP_002152231_Penicillium marneffei	PLNVQTYNKVKKVVKNARYTQNTLGQNNLLELAATPSG-----
XP_002148277_Penicillium marneffei	WINVQSQAVKKVIRYNARYTQNNLGEPNLLVVAAKAFDCNVSSSMWS
	:.** : ***:::***** **: *** :** .

Figure 14 Continued

**Figure 15**

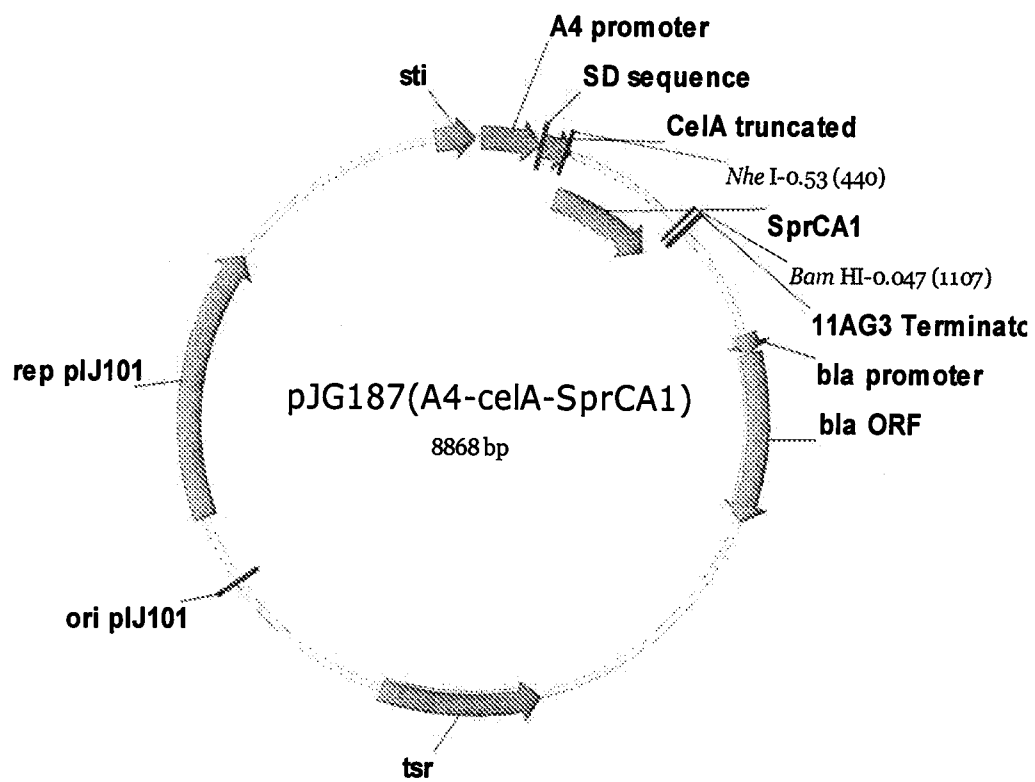
**Figure 16**

Figure 17A

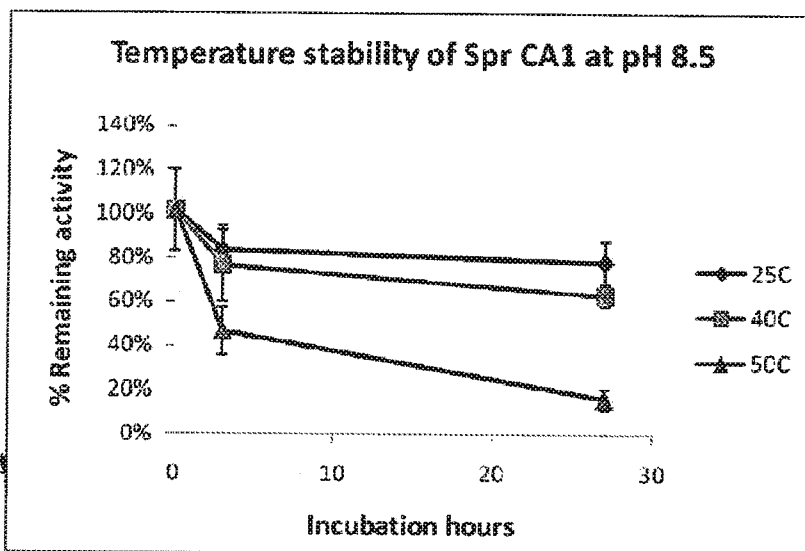


Figure 17B

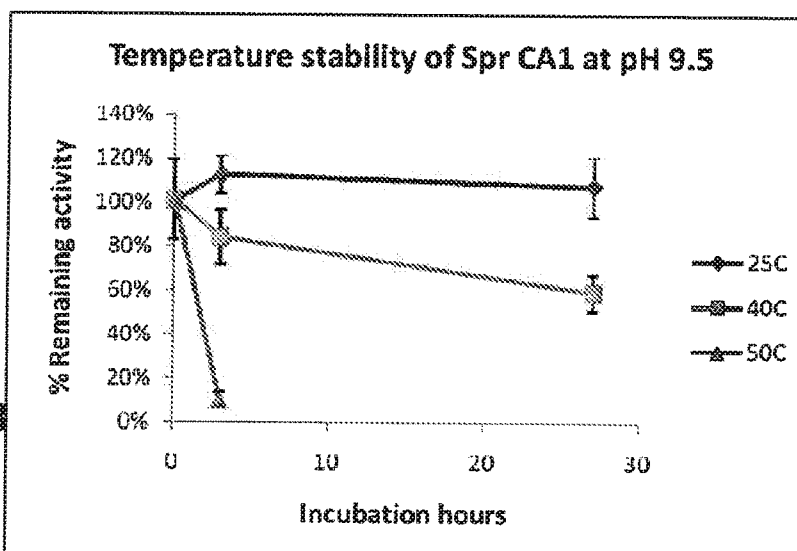
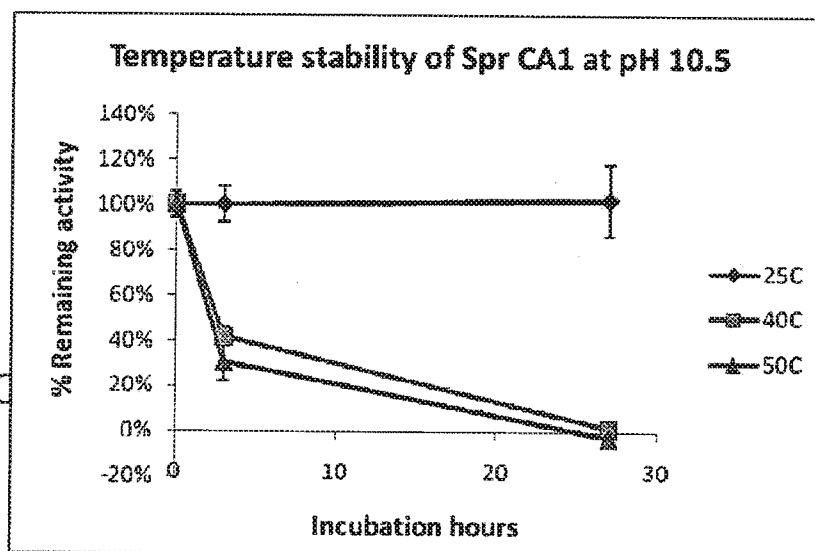
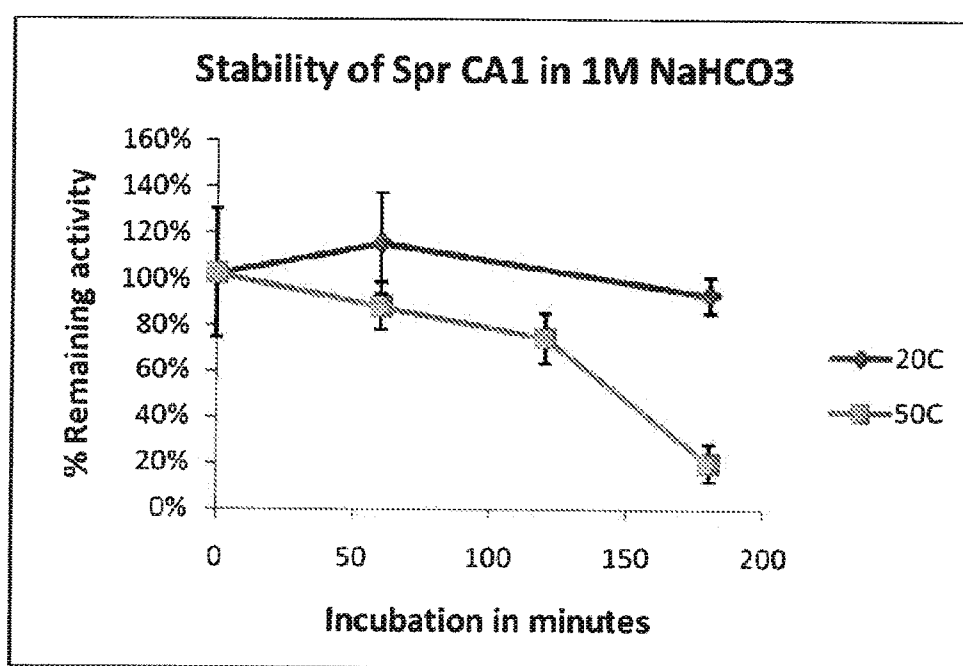


Figure 17C



**Figure 18**

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ZP_07308563_Streptomyces viridochromogene  --MNSHTTSWRGSGPEPHSEAGSRPTPGGTVRATLLRTALTGAALGAGF
ZP_04996739_Streptomyces sp. Mgl  -----M
ZP_05006186_Streptomyces clavuligerus  -----MTPSERHAAGGATPAFVSRPRRGLLRAAIIGSTVTAGAW
YP_003487983_Streptomyces scabiei  MNLDAAPEHSGDTAEATAGGADAFISPAQPPRRRSLRAALAGTAVVGGGL
ZP_06588467_Streptomyces roseosporus  -----ML
ZP_04697556_Streptomyces roseosporus  -----MRITRTPATPTTRRTLLRASAATAAGVL
ZP_06907409_SprCA1  -----MKNTFPTNSSVGGGSRRTLLRAAVAGGALASGGL
ZP_07290742_Streptomyces sp.  ---MLSNIKPSNVSPVAAAPNTAARGADSPSRRTLLRTAVAGTATAGGVL
ZP_06769567_Streptomyces clavuligerus  -----MTVNVQNRLEENVVAASHGTLRRSMLRAAVGGASALGALG

ZP_07308563_Streptomyces viridochromogene  AARPQATATTSAPSTFP-----RPGTPDEALRELAEGNRWRWAYRE
ZP_04996739_Streptomyces sp. Mgl  VSGSSAVSTRSDGGVRR-----REVTFEGAWAELAAGNGRWRTLRE
ZP_05006186_Streptomyces clavuligerus  ALGGPAASAVPRTSTGF-----RPATPEAALREHAGNQWRTPHE
YP_003487983_Streptomyces scabiei  AVGAFFANAESVSRSTPAHQTPPAHKRFTTAEELKELSQGNRRWRTLHE
ZP_06588467_Streptomyces roseosporus  AAGSSPVGAAPRTRFASA-----ARPATPAAALRELAAGNRWRTLHQ
ZP_04697556_Streptomyces roseosporus  AAGSSPVGAAPRTRFASA-----ARPATPAAALRELAAGNRWRTLHQ
ZP_06907409_SprCA1  VWAGTPASASFGPATARR-----PRPGTPAQALRELAAGNRWRTRFQ
ZP_07290742_Streptomyces sp.  ALGSAFPASATFPAPTGG-----SRPTTFEQALRELAAGNRWRTRYQ
ZP_06769567_Streptomyces clavuligerus  VLGGGTATAASPLHTPPSPASSFPTRPVTRPRAALQALLDGNRRWRSTQ

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ZP_07308563_Streptomyces viridochromogene  RHPHEDPALRRTLATAQHFFAVVLGCIDSRVPPELVFDQGLGDLMTVRSR
ZP_04996739_Streptomyces sp. Mgl  RHPDESAGLRRELVGQGNFFAVVLGCVDSDRVPPPELVFDQGLGDLTVRSA
ZP_05006186_Streptomyces clavuligerus  QHPHETHHTIRREAVSGQNHFFAVVLGCIDSRVPPELVFDQGLGDLTVRSA
YP_003487983_Streptomyces scabiei  QHPDEGYALRKALTTGQQPFALVLGCIDSRVPPELVFDQGLGDLMTVRSR
ZP_06588467_Streptomyces roseosporus  RHPHETAGVRSALVSGQSPFALILGCIDSRVPPELVFDQGLGDLMTVRSR
ZP_04697556_Streptomyces roseosporus  RHPHETAGVRSALVSGQSPFALILGCIDSRVPPELVFDQGLGDLMTVRSR
ZP_06907409_SprCA1  QHPHENSARVEELISGQEPFAVVLGCIDSRVPPELVFDQGLGDLMTVRSR
ZP_07290742_Streptomyces sp.  EHPHESPSTLRRLAKGQNFPAVVLGCVDSDRVPPPELVFDQGLGDLTVRSA
ZP_06769567_Streptomyces clavuligerus  RHPNEDPAARERAIAGQAPFAVVLGCIDSRVPPEHVFDQGLGDLTVRSA

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Figure 19

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ZP_07308563_Streptomyces viridochromogene      GEVLDEAVLGSVAYGVLELAIPLVVVLGHQSCGAVRAAVQAEQSGERLPA
ZP_04996739_Streptomyces sp. Mgl                GEVLDEAVVGSVAYGVLELGIPLVVVLGHQACGAVAAVHAETCHGELPG
ZP_05006186_Streptomyces clavuligerus           GQVLDEAVLGSVAYGVLELDIPLVVVLGHQSCGAVAAVHADETGAELPA
YP_003487983_Streptomyces scabiei               GEVLDDQSVLGSVKYGVLELNIPLVVVLGHQSCGAVKAAVAVDESSEELPS
ZP_06588467_Streptomyces roseosporus            GEVLDEAVLGSIQYGVLELGIPLVVVLGHQSCGAV----HAEETGESLPA
ZP_04697556_Streptomyces roseosporus            GEVLDEAVLGSIQYGVLELGIPLVVVLGHQSCGAV----HAEETGESLPA
ZP_06907409_SprCA1                              GEVLDEAVLGSVAYGVLELDIPLVMVLGHQSCGAVTAAVHAEETGEELPA
ZP_07290742_Streptomyces sp.                   GEVLDEAVLGSIAYGVLELKIPLVLVLGHQSCGAVKAAVHADETGERLPA
ZP_06769567_Streptomyces clavuligerus           GQVLDESIVGSVEYGVVALRIPLVVVLGHQSCGAVKSAIEIEQTGEQLPG

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ZP_07308563_Streptomyces viridochromogene      HMQYLVDDQIGPAIDHGVGDGARI DATVSANVRLVRSRLAAEPELAARVAD
ZP_04996739_Streptomyces sp. Mgl                PLRYLAGQIRPSIDRTLRGDACVDAAVTANVRRVAARLAAQAE MAGRIAA
ZP_05006186_Streptomyces clavuligerus           HIQYIAAEIRPAIDRSVQGDARIDATVSAQVREVRSELAAEPDLAPHIAA
YP_003487983_Streptomyces scabiei               GIQYIAGEIAPAIIDKSVTGDARVAATIDANVRLVRSKVVAQPDVAAHLKA
ZP_06588467_Streptomyces roseosporus            HIQYVAEQIRPAIVHGQHGCDARVDATVSAQVRLVRSRLARETDLASKVAA
ZP_04697556_Streptomyces roseosporus            HIQYVAEQIRPAIVHGQHGCDARVDATVSAQVRLVRSRLARETDLASKVAA
ZP_06907409_SprCA1                              HIQYIADRIRPAIDHSQEGAAPVOSTITRNQVMTRELLAQEPDLAARIAA
ZP_07290742_Streptomyces sp.                   HIQYLADQINPAIDRTHGCDARVRATIDAHARSTRDELAAEPDLARAVSA
ZP_06769567_Streptomyces clavuligerus           SIQYIAERIWPIDQTQOGDARLNATTDANALMIRDQLAALPTIATRIAA

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ZP_07308563_Streptomyces viridochromogene      GRLAIVGARVELTTQTVHRVA
ZP_04996739_Streptomyces sp. Mgl                GKLAIVGARVELAGQWVHQVG
ZP_05006186_Streptomyces clavuligerus           GRLAITGARVELTSQLVHQLA
YP_003487983_Streptomyces scabiei               GKVAVVGARYDLTTQRVHLLK
ZP_06588467_Streptomyces roseosporus            GELAIVGARVELSTELVHRVG
ZP_04697556_Streptomyces roseosporus            GELAIVGARVELSTELVHRVG
ZP_06907409_SprCA1                              GKLAIVGARVELSSQLVHTVA
ZP_07290742_Streptomyces sp.                   GKLAVAARYDLHDQRVSTLN
ZP_06769567_Streptomyces clavuligerus           GQLSVVSARYELTDQSVRLLP

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Figure 19 Continued

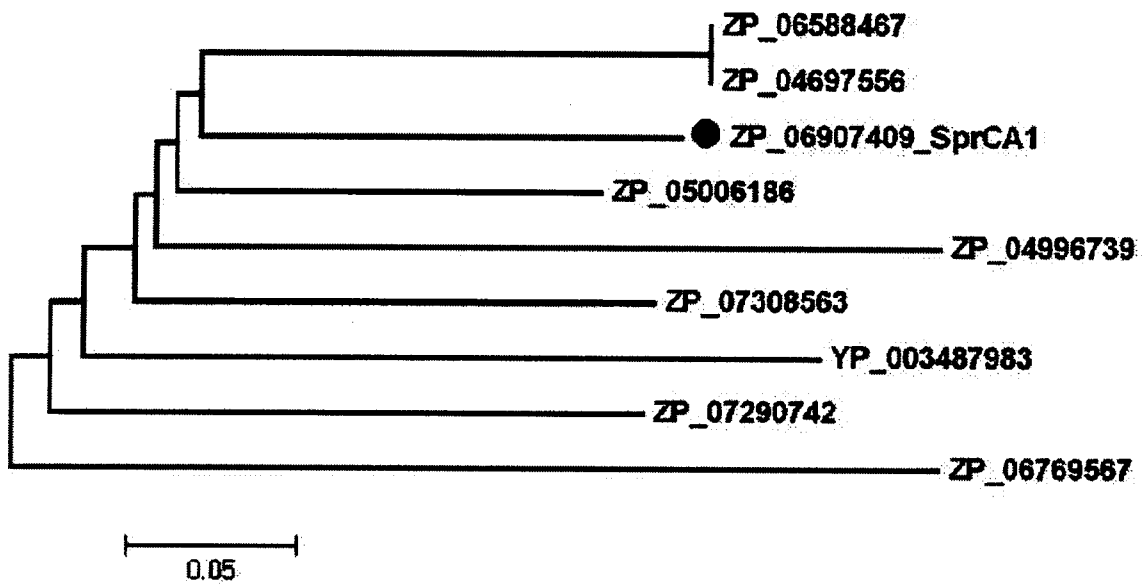


Figure 20

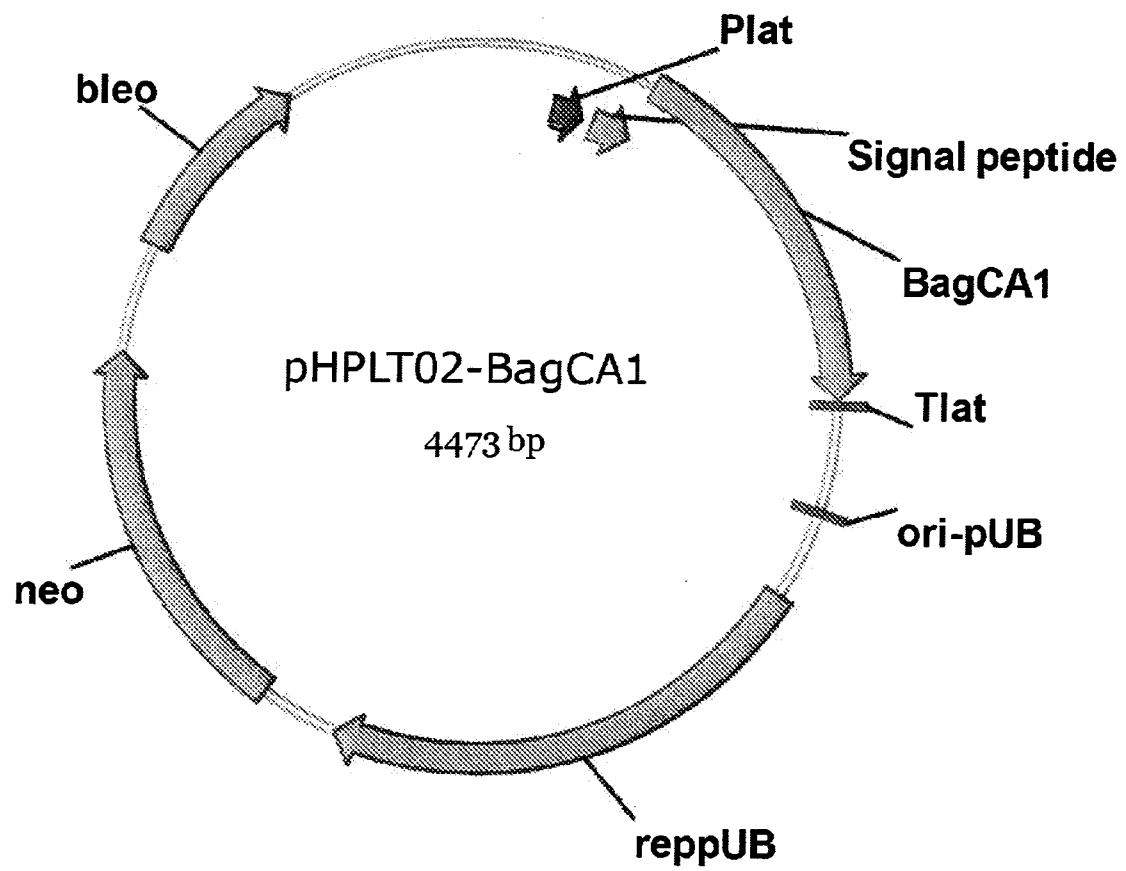
**Figure 21**

Figure 22A

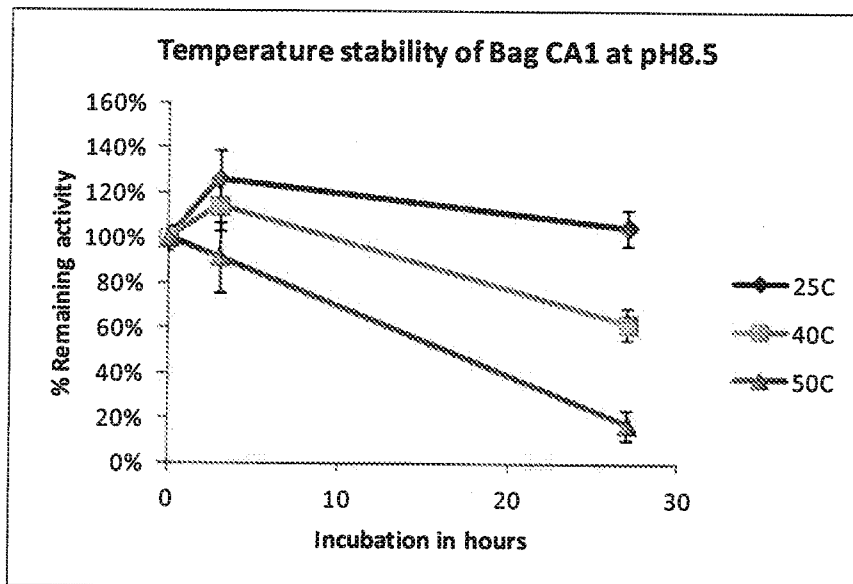


Figure 22B

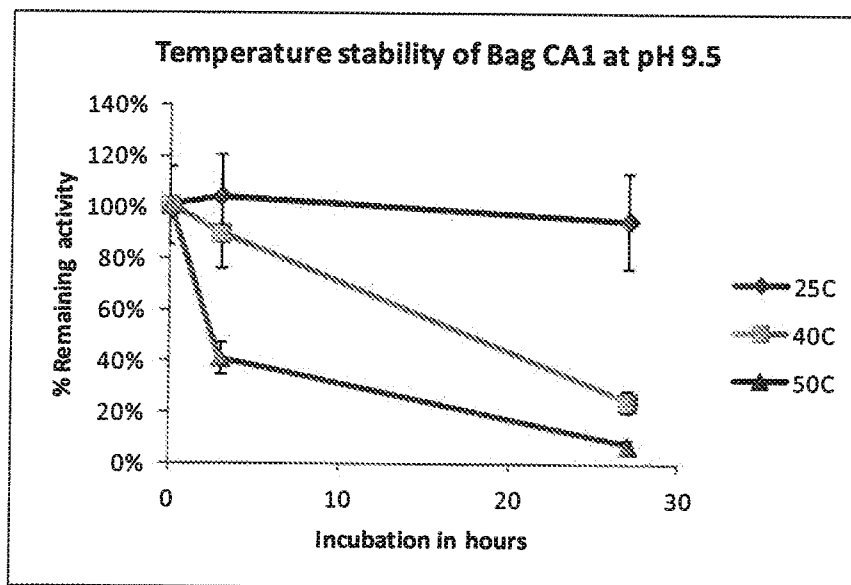
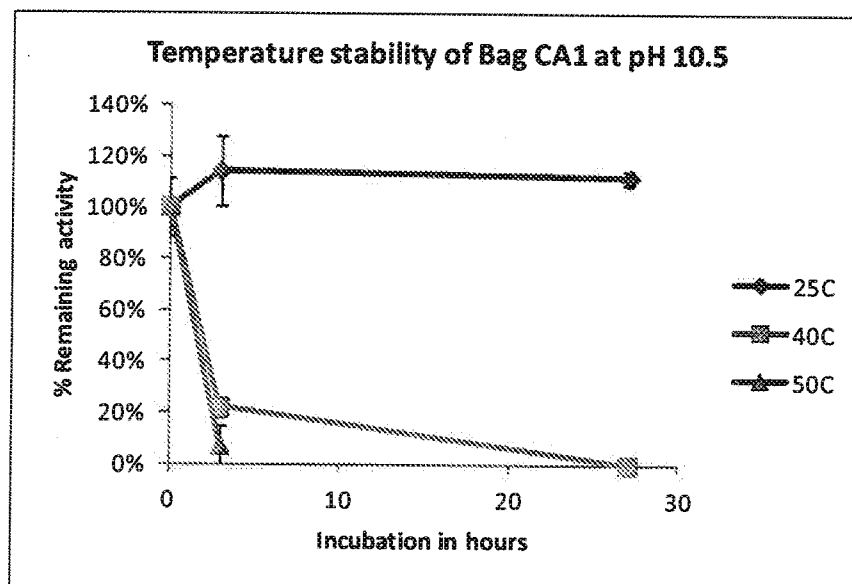
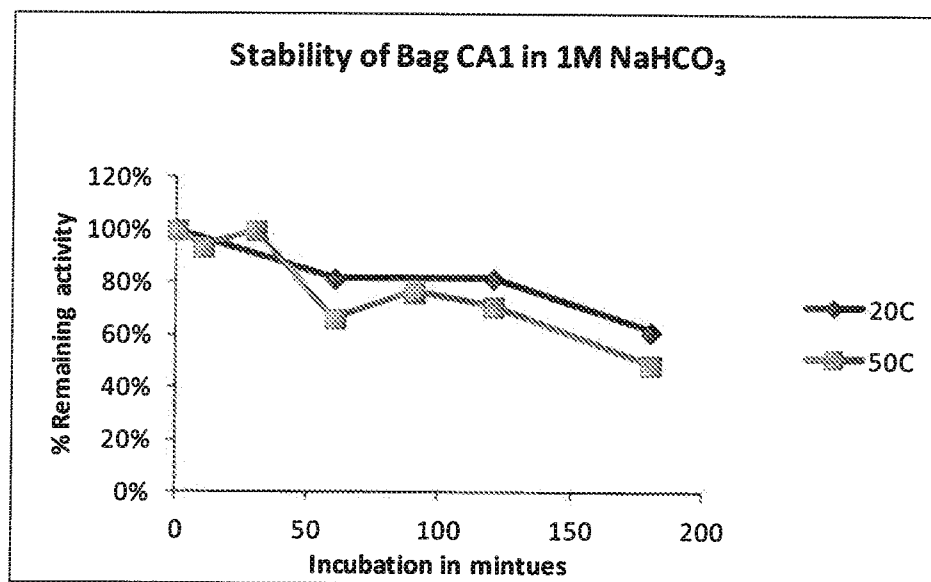


Figure 22C



**Figure 23**

NP_241226_Bacillus_halodurans	-----
BagCA1_mature	-----GDEEVNSLNGSGTHEEAIKDEH
YP_419944_Magnetospirillum_magneticum	-----MDRRSFLKGATGAACLCGTCGGSIAEAVAAE
ZP_08403178_Rubrivivax_benzoatilyticus	-----PGDAPAARKPAPRPRPEVPAP
YP_004645315_Paenibacillus_mucilaginosus	-----
YP_003944556.1_Paenibacillus_polymyxa	-----
ZP_04248491_Bacillus_cereus_Rock1-3	-----MKGLKFVYKTPLILTMSVVLGMCNTAQEAPQKQSTKE
ZP_04209878_Bacillus_cereus_Rock4-18	-----MGCNTAQEAPHKQSTKE
YP_003428428_Bacillus_pseudofirmus	MKRKSYLLVAASLALAAEQTETAPVETPPTDSTEPSDETQEESTEE
BAK17997_Solibacillus_silvestris	-----
YP_176677_Bacillus_clausii	-----

NP_241226_Bacillus_halodurans	-----CAQGEEQSPINLDKAE--AVDTDTEI
BagCA1_mature	EGHWSYTGETGPTHWGSLDASYELCEQEQQSPINIETDE--VTTTDTTHI
YP_419944_Magnetospirillum_magneticum	GAHWAYEGHGGPKWGMLSPEYAACSMGREQSPVDLTRP---IAAIIGDP
ZP_08403178_Rubrivivax_benzoatilyticus	EIHWNVTGEGGPARWGALRPEFGLCARGQRQSPIDIRGG---IAVDLEEI
YP_004645315_Paenibacillus_mucilaginosus	-----DQTF AACANGTEQSPVDIELTQTQKVDKTAVQV
YP_003944556.1_Paenibacillus_polymyxa	-----DFVACGNGQEQQSPINIEHSHLEASHTQQPL
ZP_04248491_Bacillus_cereus_Rock1-3	NTQWSYKGTGPEHWGELKPEYNMCLNGQEQQSPIDIKTEQIKSTVDNNLL
ZP_04209878_Bacillus_cereus_Rock4-18	NTQWSYEGTTGPEHWGELKPEYKICLNGQEQQSPIDIKTEQIKSTVDNNLL
YP_003428428_Bacillus_pseudofirmus	LTEWSYEGESGPEHWGHLHASYSACVDGSEQSPINIDLAEMEANQQIEEI
BAK17997_Solibacillus_silvestris	-----NVEFPEVKADGNLKGNN
YP_176677_Bacillus_clausii	-----NLFYDREQTSKWNW

NP_241226_Bacillus_halodurans	QVHYEPSAFTIKNNGHTIQAETTS DG-NTIEIDGKEYTLVQFHFHPSEH
BagCA1_mature	SIAYQPSFPAIENNGHTIQANALTED-NTISIEGENYQLIQFHFHPSEH
YP_419944_Magnetospirillum_magneticum	MAAWRPVPLRVQNNNGHTIQVDCSGGG--TLMLDGKSYDLLQFHFHPSEH
ZP_08403178_Rubrivivax_benzoatilyticus	VFDYRPAGFAVL DNGHTVQVNVGPGN--VIAVGRRYELQQFHFHRPSEE
YP_004645315_Paenibacillus_mucilaginosus	ELHYQPSAFTLMNNGHTIQANAAAGNGNTITVDGTDYTLAQMHHFHPSEN
YP_003944556.1_Paenibacillus_polymyxa	QVHYSTTKVSILNNGHTVQVNAASPS-NDIVVDGTFKTLKQFHFHPSEH
ZP_04248491_Bacillus_cereus_Rock1-3	QINYQPISFSIKNNGHSIEGKANSSD-DYLT LGENRYTLKQFHFHTPSEH
ZP_04209878_Bacillus_cereus_Rock4-18	QINYQPISFSIKNNGHSIEGKANSSD-NYLT LGENRYTLKQFHFHTPSEH
YP_003428428_Bacillus_pseudofirmus	DIQYEPASFSLVNNNGHTIQKNAVDEN-NAITLDGQEYQLVQFHFHTPSEH
BAK17997_Solibacillus_silvestris	EIHYEPTPYTLENNNGHTIQANATTES-NHIIIEDNEYKLSQFHFHTPSEH
YP_176677_Bacillus_clausii	AFSYSEAAFSVENNNGHTIQANVENEDAGGLEINGEAYQLIQFHFHTPSEH
	: : :***: : . : : : * *:*** **.

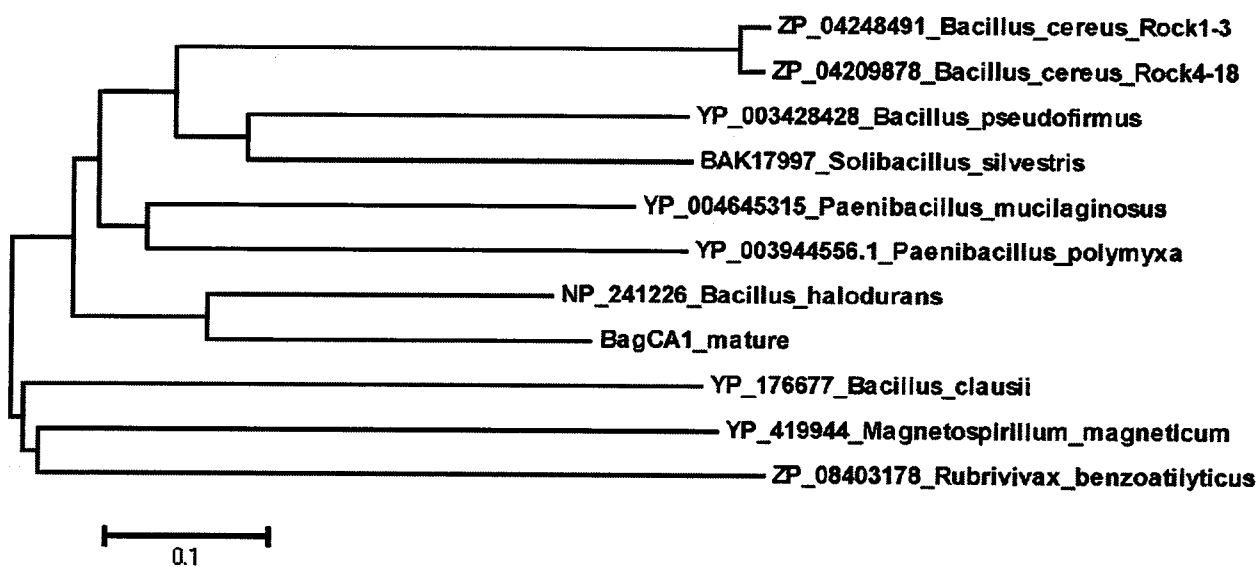
Figure 24

NP_241226_Bacillus_halodurans	EMEGKNLDMELHFVHKNEDELAVLGVLKAGEENEELAKLWSKLPAAET
BagCA1_mature	QKNGEHLDMELHFVHQNEGELAVLGVLMEEGEVNDALAEWAEMPQEEM
YP_419944_Magnetospirillum_magneticum	TVDGAFDMECHFEVHKAADGGLAVLGVMIAKGAANPALEAIWQVMPAKAG
ZP_08403178_Rubrivivax_benzoatilyticus	RIDGRQSEMVAHLVHRDPEGR LAVVAVLLQRGEEQPVVQSVWNNLPLEKH
YP_004645315_Paenibacillus_mucilaginosus	QINGKNFEMEGHLVHKNDGGLAVVGFLMTAGKENKPLAEMWSKLPKQET
YP_003944556.1_Paenibacillus_polymyxa	QIDGKNAEMELHFVHQSDTGSTAVLGVLIOGSKENKAFNRIWSKLPKDIS
ZP_04248491_Bacillus_cereus_Rock1-3	QFEGKHADMELHLVHQNDQGQLAVVGIMIKEGQKNEGFAAMWQNLPHRKN
ZP_04209878_Bacillus_cereus_Rock4-18	QFEGKHADMELHLVHQNDQGQLVVGIMIKEGQKNEGFAAMWQNLPHRKN
YP_003428428_Bacillus_pseudofirmus	QFNGEHYDMELHLVHQDINGNLAVLGVMIEEGAENEELAPAWGELPEEET
BAK17997_Solibacillus_silvestris	QFNGQNYDMELHLVHSDKDKLAVIGLMIQEGNENKQFASMMWNLPTDKT
YP_176677_Bacillus_clausii	TIETSFPMELHLVHANHAGDLAVLGVLMMENDHEGIEAVWEVMPPEEG
	: * *: * . *: . . : * : . * : *

NP_241226_Bacillus_halodurans	EENISLDESIDLNALLPESKEGFHYNGSLTTPPCSEGVKWTVLSEPTVS
BagCA1_mature	DETIELTDAIDLNALLPSSHEGFHYGGSALTTPPCTEGVKWVVLEKTI SVS
YP_419944_Magnetospirillum_magneticum	EAATA-TSMLDASMLLPKDPVTFRYAGSLTTPPCTEVVQWVVYRQAITAS
ZP_08403178_Rubrivivax_benzoatilyticus	DEARA-SGAIDLQALLPADRGYYTYMGSLTTPPCTEGLWIVMRQPVAVS
YP_004645315_Paenibacillus_mucilaginosus	KEDVKLEQPVLDLPGVLPSTAHAFRYEGSLTTPPCTSEHVKWIVLADPIEVS
YP_003944556.1_Paenibacillus_polymyxa	QEAV-LDEDVNLAALLPKDLHSVRYNGSLTTPPCTEHNWTVLEQPIEMS
ZP_04248491_Bacillus_cereus_Rock1-3	IKADV-QHTIDIKQILPSDHSFRYMGSLTTPPCTENVQWIVMKQTIEMS
ZP_04209878_Bacillus_cereus_Rock4-18	IKADV-QHTIDIKQILPSDYSSFRYMGSLTTPPCTENVQWIVMKQTIEMS
YP_003428428_Bacillus_pseudofirmus	ENEVALEEPINLQNLPPDQSSPHYNGSLTTPPCTEEVKWIVFKEPIQKS
BAK17997_Solibacillus_silvestris	AKGNSEKHIIDLQALLPENETTFQYAGSLTTPPCTEEVQWIVFEQPIEMS
YP_176677_Bacillus_clausii	TAAYS--ISLDPNLFPESVTAYQYDGSALTTPPCTSEGVKWTVLNDTISIS
	:: ::* * *****: * * * * . . : *

NP_241226_Bacillus_halodurans	QEQIDAFAEIFPDNHRPVQPNDRDVYDVITE
BagCA1_mature	QEQIDTFAEIFPTNNRPVQPNDRHVYEVAD
YP_419944_Magnetospirillum_magneticum	AEQLAAFAKLFPPNNARPVQPLNRRKLLLDVM-
ZP_08403178_Rubrivivax_benzoatilyticus	QEQIDIFARLYPMNARPVQPSGRLIKQSR--
YP_004645315_Paenibacillus_mucilaginosus	KEQIEAFAAIFPDNHRPVQPLNQRTVVSN---
YP_003944556.1_Paenibacillus_polymyxa	ADQIKQFAAIFPDNHRPVQQLGTRELKADK--
ZP_04248491_Bacillus_cereus_Rock1-3	KKQIKVFHKLFPPTNNRPVQPINGRAVL-----
ZP_04209878_Bacillus_cereus_Rock4-18	KKQIKVFHKLFPPTNNRPVQPINGRAVL-----
YP_003428428_Bacillus_pseudofirmus	AEQIQAFQEIYEENHRPVQPLNERG-----
BAK17997_Solibacillus_silvestris	KAQIKAFQKIFPDNHRPVQPINEREINKSGE-
YP_176677_Bacillus_clausii	ETQLDAFRDIYPQNYRPVQELGDREIGFHYH-
	*: * :: * ***** . *

Figure 24 Continued

**Figure 25**

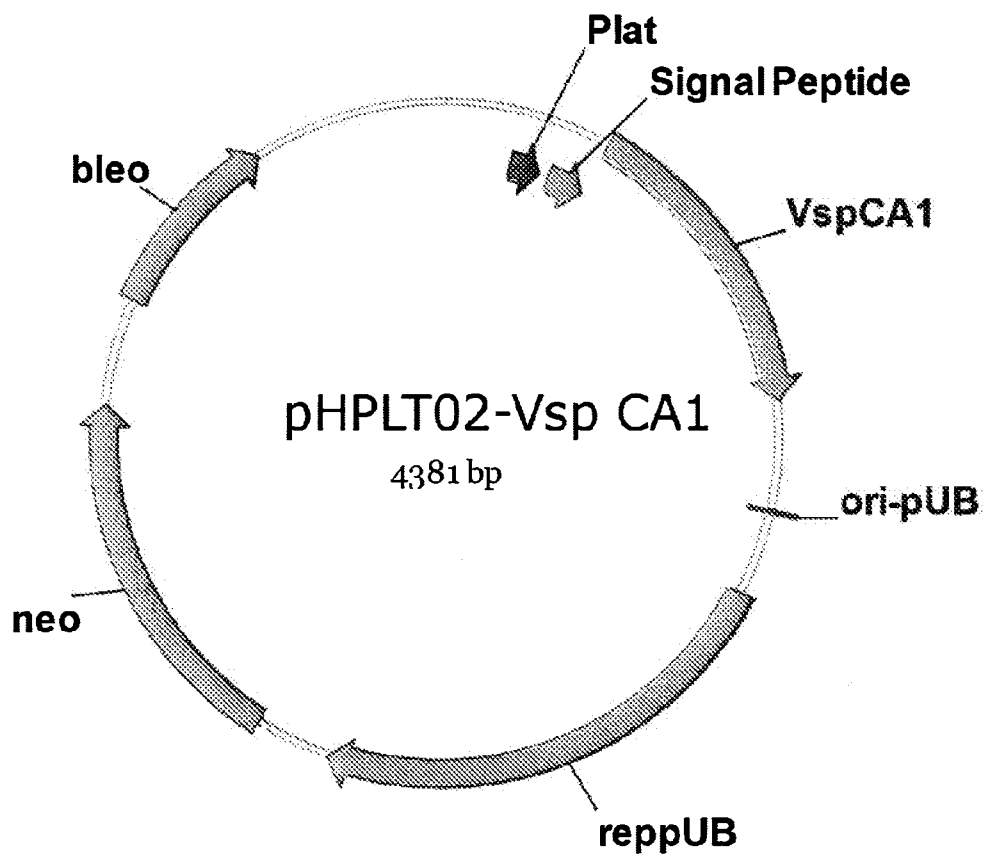
**Figure 26**

Figure 27A

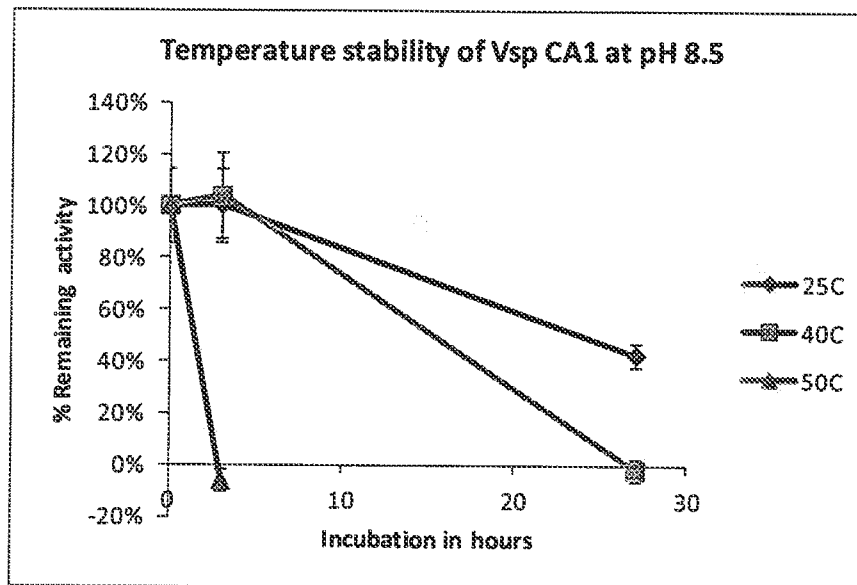


Figure 27B

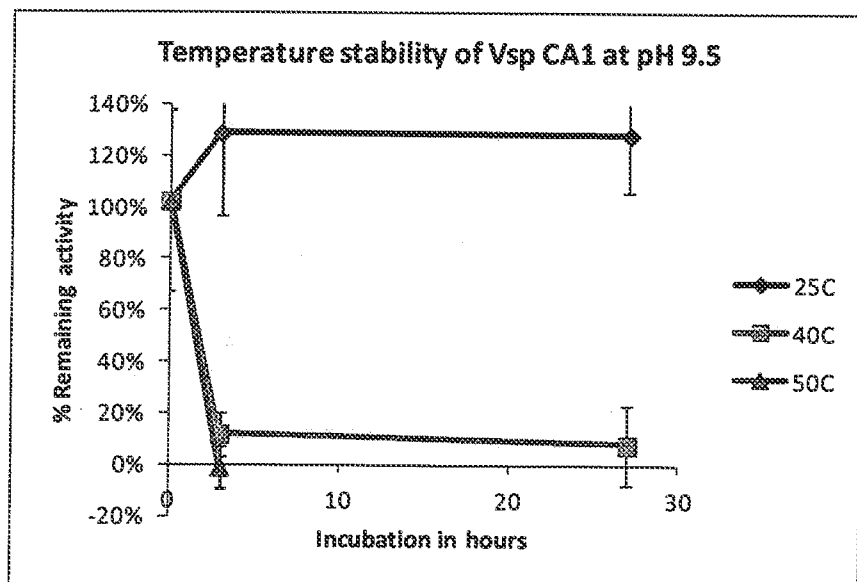
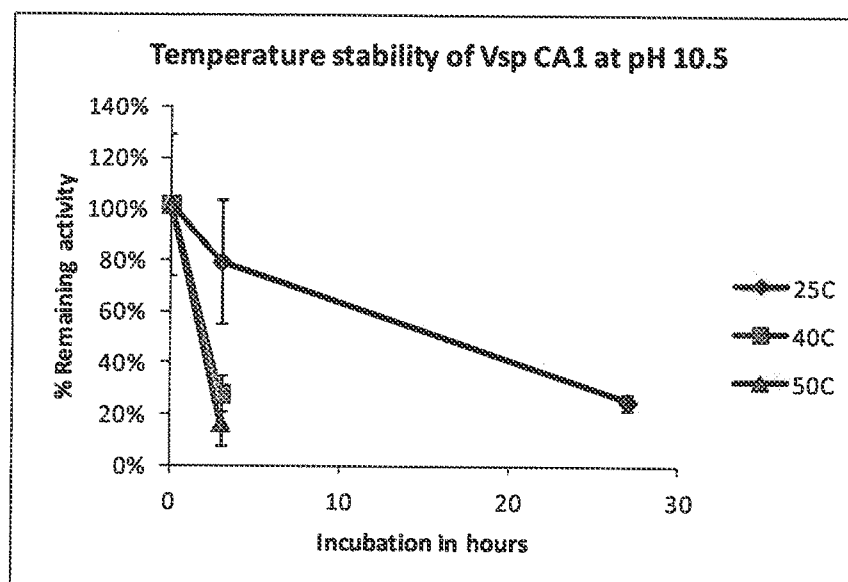


Figure 27C



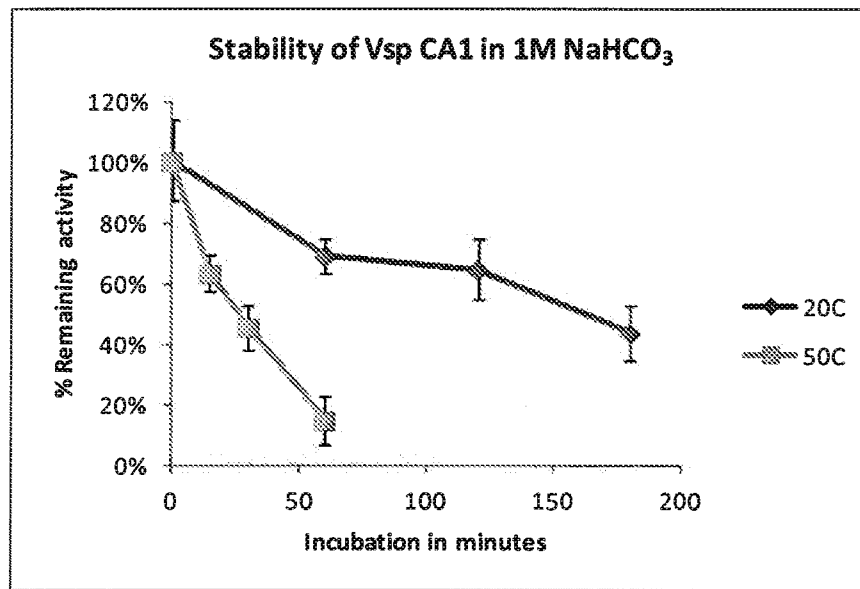
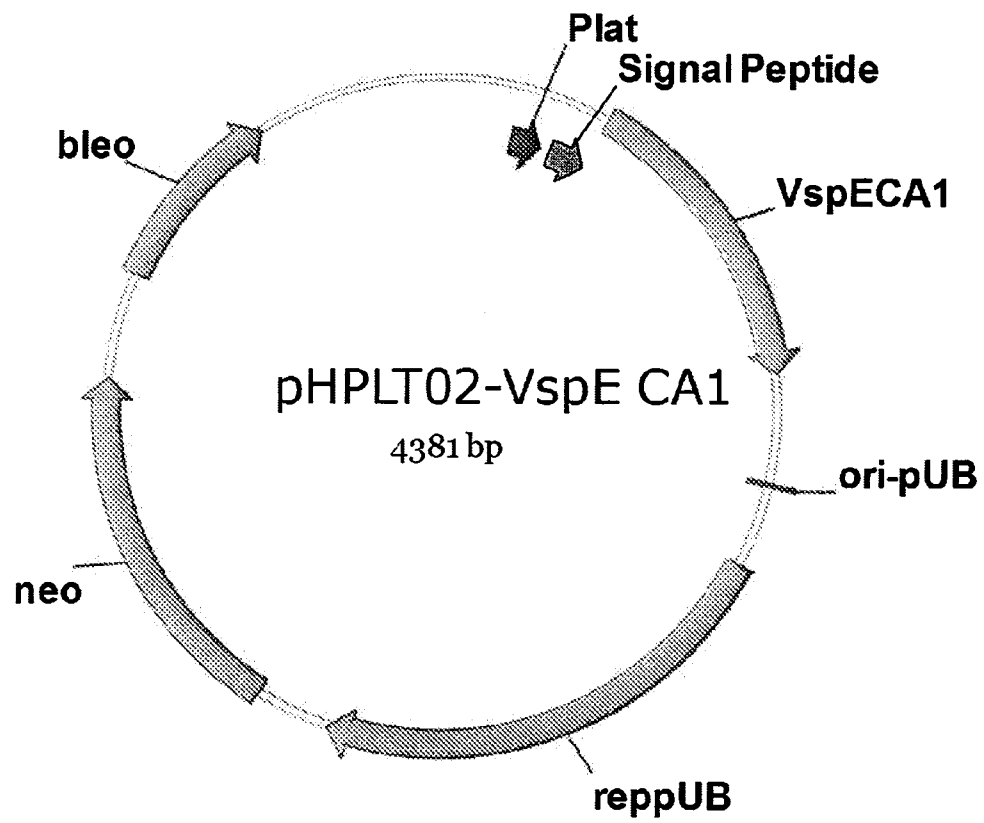
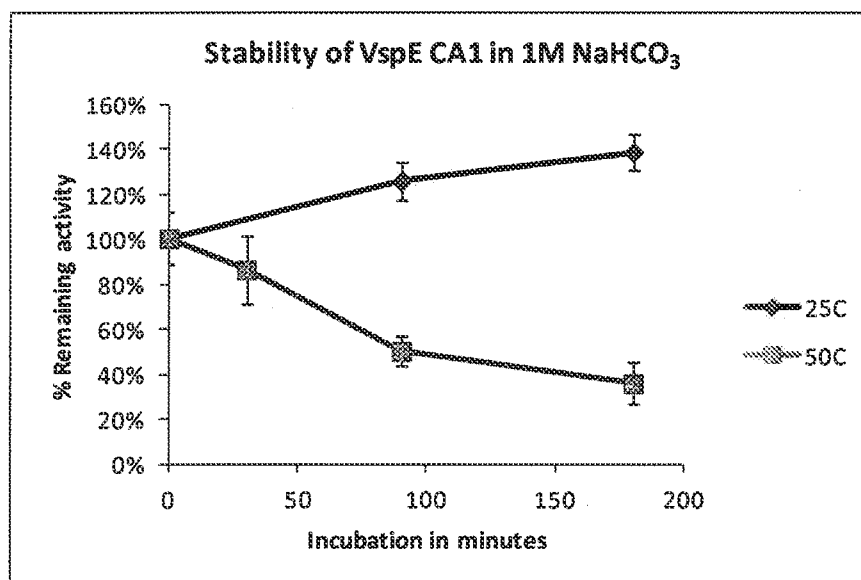
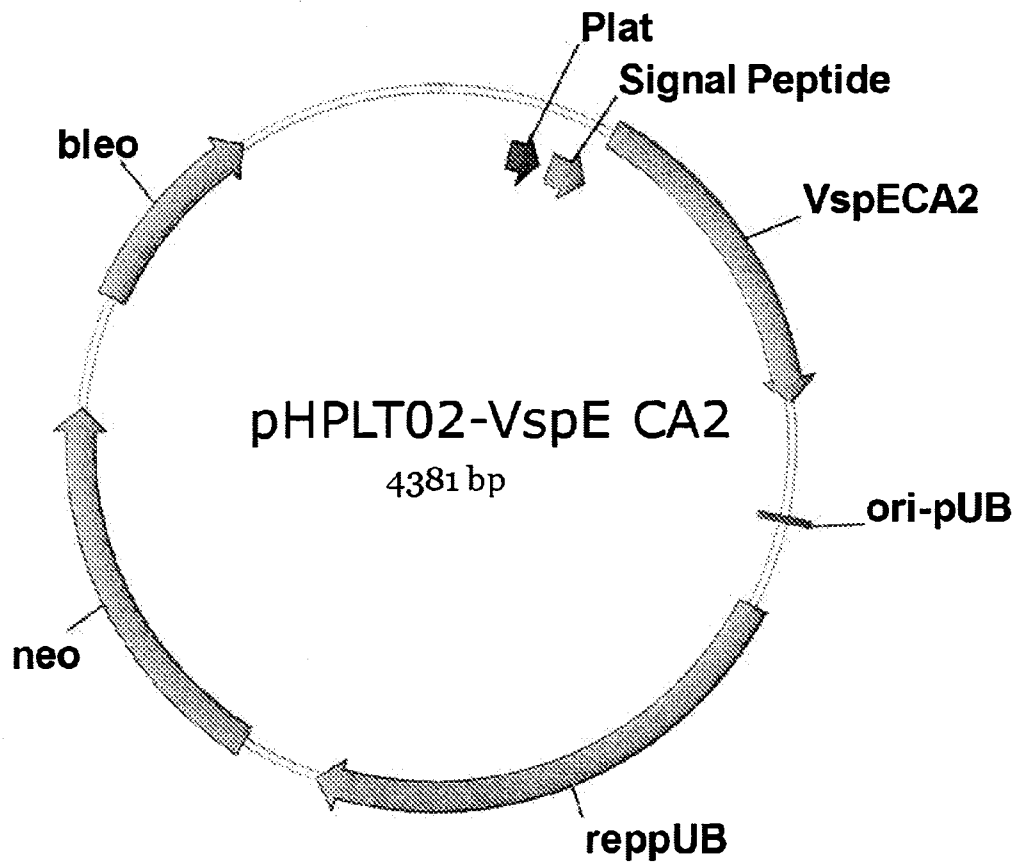
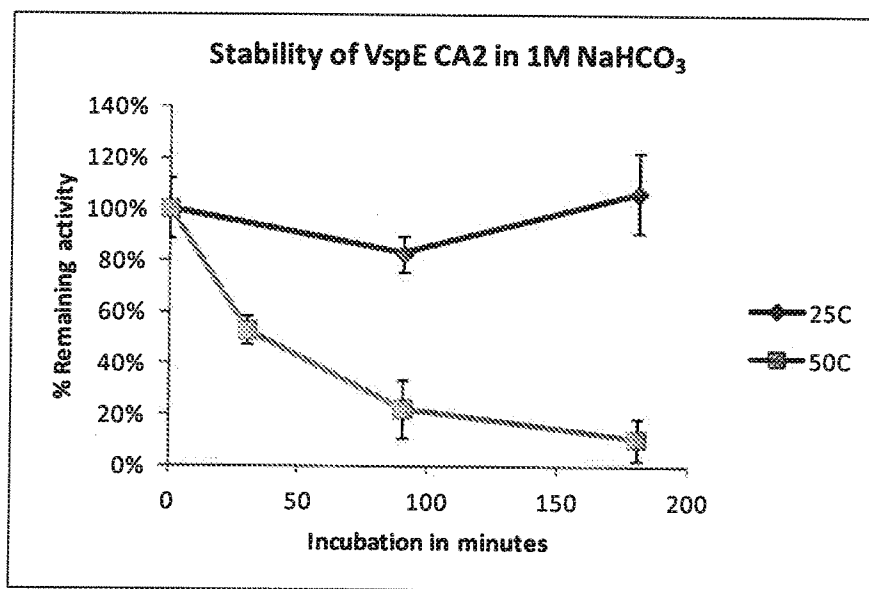
**Figure 28**

Figure 29

**Figure 30**

**Figure 31**

**Figure 32**

**Figure 33**