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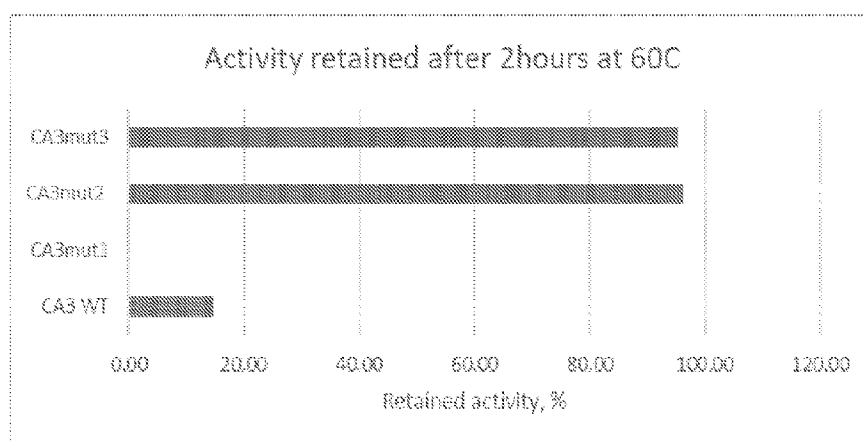


FIG. 8

(57) Abstract: Disclosed herein are compositions and methods for making and using improved carbonic anhydrases for novel, sustainable, and low energy CO₂ waste gas scrubbing technologies that are also transformational carbon capture technologies. Embodiments of methods, systems and compositions disclosed herein include, but are not limited to, non-aqueous solvents, advanced membranes, sorbents, and cryogenic systems that significantly reduce the cost of CO₂ capture from coal and natural gas-fired power plants and industrial facilities. Methods disclosed herein reduce the energy and cost required for CO₂ separation and can be applied for both pre-combustion and post-combustion CO₂ capture.



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ENGINEERED THERMOSTABLE CARBONIC ANHYDRASE ENZYMES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority under 35 U.S.C. § 119 to U.S. provisional patent application no. 63/151,506 filed on 19Feb2021 and 63/174,337 filed on 13Apr2021, the contents of which are hereby incorporated in their entirety.

CONTRACTUAL ORIGIN

[0002] The United States Government has rights in this invention under Contract No. DE-AC36-08GO28308 between the United States Department of Energy and the Alliance for Sustainable Energy, LLC, the Manager and Operator of the National Renewable Energy Laboratory.

SEQUENCE LISTING

[0003] This application contains a Sequence Listing which has been submitted via EFS-web and is hereby incorporated by reference in its entirety. The ASCII copy as filed herewith was originally created on 21Feb2022. The ASCII copy as filed herewith is named NREL_PCT_20-137_ST25.txt, is 58 kilobytes in size and is submitted with the instant application.

BACKGROUND

[0004] Energy demand continues to rise along with CO₂ emissions. Carbon Capture and Storage (CCS) plays a significant role in reducing CO₂ emissions produced from the use of fossil fuels in electricity generation and industrial processes. Bioenergy with Carbon Capture and Storage (BECCS) combines the use of biopower with greenhouse gas mitigating technology to produce energy with net-negative emissions. However, today's capture technologies are not cost-

effective. Most current CCS processes rely on carbon scrubbing of flue gases with solvents like monoethanolamine (MEA) which requires energy intensive heating and cooling of the MEA to capture and release the CO₂ generated in combustion. In addition, the solvent is corrosive and suffers degradation by other species present in gas mixtures. There is a need for alternative novel scrubbing techniques that incorporate biological solutions for capturing CO₂ to improve the cost of carbon capture.

[0005] Carbonic anhydrases (CAs) are an example of convergent evolution where at least five distinct families of enzymes catalyze the same reaction but do not share significant sequence similarity or fold. Most but not all families of CA have been characterized structurally.

[0006] The chemical and enzymatic properties of CAs, like specific activity, thermal stability, and chemical stability vary greatly and have been previously targeted for improvement in industrial applications.

SUMMARY

[0007] In an aspect, disclosed herein is a non-naturally occurring carbonic anhydrase comprising at least one mutation that results in the substitution of at least one cysteine for at least one amino acid in a naturally occurring carbonic anhydrase; and wherein the non-naturally occurring carbonic anhydrase has increased activity at a temperature of greater than about 60 degrees Celsius when compared to the naturally occurring carbonic anhydrase. In an embodiment, the non-naturally occurring carbonic anhydrase has increased activity that is for more than about 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 24 hours, 44 hours, 48 hours, and 92 hours. In an embodiment, the non-naturally occurring carbonic anhydrase has increased activity that is at a temperature greater than 65, 70, 75, 80, 85 or 90

degrees Celsius. In an embodiment, the non-naturally occurring carbonic anhydrase has a nucleotide sequence encoding the non-naturally occurring carbonic anhydrase that comprises a sequence that is greater than 70% identical to a sequence selected from the group consisting of SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 22, SEQ ID NO: 24 and SEQ ID NO: 26. In an embodiment, the non-naturally occurring carbonic anhydrase has an amino acid sequence that is greater than 70% identical to a sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 21, SEQ ID NO: 23 and SEQ ID NO: 25.

[0008] In an aspect, disclosed herein is a method for CO₂ separation and CO₂ capture comprising the step of reacting CO₂ with a non-naturally occurring carbonic anhydrase comprising at least one mutation that results in the substitution of at least one cysteine for at least one amino acid in a naturally occurring carbonic anhydrase; and wherein the non-naturally occurring carbonic anhydrase has increased activity at a temperature of greater than about 60 degrees Celsius when compared to the naturally occurring carbonic anhydrase. In an embodiment, the method contains the step of reacting CO₂ with non-naturally occurring carbonic anhydrase is for more than about 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 24 hours, 44 hours, 48 hours, and 92 hours. In an embodiment, the method contains the step of reacting CO₂ with the non-naturally occurring carbonic anhydrase is at a temperature greater than 65, 70, 75, 80, 85 or 90 degrees Celsius. In an embodiment, the the non-naturally occurring carbonic anhydrase comprises a nucleotide sequence that is greater than 70% identical to a sequence selected from the group consisting of SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 22, SEQ ID NO: 24 and SEQ ID NO: 26. In an embodiment, the non-naturally occurring carbonic anhydrase comprises an amino acid sequence that is greater than 70% identical to a sequence

selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 21, SEQ ID NO: 23 and SEQ ID NO: 25.

[0009] In an aspect, disclosed herein is a system for CO₂ separation and CO₂ capture comprising non-naturally occurring carbonic anhydrases comprising at least one mutation that results in the substitution of at least one cysteine for at least one amino acid in a naturally occurring carbonic anhydrase; and wherein the non-naturally occurring carbonic anhydrase has increased activity at a temperature of greater than about 60 degrees Celsius when compared to the naturally occurring carbonic anhydrase; and wherein the system further comprises a support wherein the with the non-naturally occurring carbonic anhydrases are immobilized to the support; and wherein the non-naturally occurring carbonic anhydrases are contacted with CO₂. In an embodiment, the non-naturally occurring carbonic anhydrase has increased activity for more than about 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 24 hours, 44 hours, 48 hours, and 92 hours. In an embodiment, the non-naturally occurring carbonic anhydrases react with CO₂ at a temperature greater than 65, 70, 75, 80, 85 or 90 degrees Celsius. In an embodiment, the CO₂ results from the combustion of fossil fuels or biomass. In an embodiment, the system further comprises a carbon capture unit wherein the carbon capture unit comprises an immobilized biocatalyst comprising an amino acid sequence that is greater than 70% identical to a sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 21, SEQ ID NO: 23 and SEQ ID NO: 25.

[0010] Other objects, advantages, and novel features of the present invention will become apparent from the following detailed description of the invention when considered in conjunction with the accompanying drawings.

DESCRIPTION OF THE DRAWINGS

[0011] FIGs. 1A and 1B depict a dimer of PmaCA. Two protein molecules are shown in cartoon representation and colored grey and green. Blue balls represent zinc ion located at the active sites of each molecule. FIG. 1A, frontal view. FIG. 1B, view from above, rotated 90 degrees.

[0012] FIGs. 2A and 2B depict a TaCA tetramer. FIG. 2A - Four protein molecules are shown in cartoon representation and colored as green, magenta, orange, and cyan. Blue balls represent zinc ion located at the active sites of each molecule. FIG. 2B - zoom-in on the disulfide bonds connecting protein molecules in the tetramer in a crisscross fashion.

[0013] FIG. 3 depicts a superimposition of PmaCA (grey), SspCA (cyan), SazCA (yellow), LogaCA (green), and TaCA (magenta). Proteins are superimposed using one protein chain for each (upper right). The fold is conserved, and the dimerization interface is also conserved.

[0014] FIGs. 4 depicts mutant 1: Gly200Cys, Asn236Cys. View from above. Two disulfides connecting molecules in the dimer are shown as spheres.

[0015] FIG. 5 depicts mutant 2, frontal view: Ala61Cys (makes disulfide with the same Ala61Cys from the 2nd molecule), one disulfide connecting molecules in the dimer is shown as spheres.

[0016] FIG. 6 depicts mutant 3: Ser189Cys, Ala237Cys, view from above. Two disulfides connecting molecules in the dimer are shown as spheres.

[0017] FIG. 7 depicts a sequence alignment for TaCA, PmaCA, LogaCA, SspCA, SazCA and PmaCA mutants 1, 2, and 3. Positions of point mutations are boxed in red for mutant 1, blue for

mutant 2, green for mutant 3 indicating where these mutations should be introduced in other CAs.

[0018] FIG. 8 depicts activity retained after PmaCA WT and mutants incubation at 60 °C for 2 hours.

[0019] FIG. 9 depicts activity retained after PmaCA WT and mutants incubation at 90 °C. The proteins were induced and expressed at 35 °C and 45 °C temperatures. Mutants 1, 3, and 2+3 combo did not have high broth activity from the beginning, so the activity of these samples quickly dropped below the method detection range. Despite sharp activity decline in the first hour for the Mutant 2, in the long run (2-48h range) this mutant retained more activity than the WT.

[0020] FIG. 10 depicts activity retained after PmaCA WT and mutants incubation at 90 °C. The proteins were induced and expressed at 35 °C and 45 °C temperatures as in Experiment 2. Protein containing broths were concentrated to boost the initial absolute activity numbers. Mutants 1, 3, and 2+3 combo did not have high broth activity from the beginning, so the activity of these samples quickly dropped below the method detection range. At 1 hour mutant 2 already retained higher activity than WT enzyme and in interval 2-72 hours mutant 2 retained significant margin above WT activity. The broth for PmaCA WT enzyme expressed at 45 °C was treated differently from other broths. It was overconcentrated first and then diluted with distilled water to reach the absolute activity levels comparable to other broths.

[0021] FIG. 11 depicts activity retained after SazCA WT and mutants 2, 3, and 2+3 combination incubation at 90 °C. Proteins were induced and expressed at 45 °C. Initial sharp drop of activity within 1st hour could be attributed to the degradation of the portion of a protein that was not able to fold correctly. Since mutant 2 has one single point mutation, mutant 3 has

two single point mutation and mutant 2+3 has three single point mutations, without being limited by theory, it is possible that negative effects from the mutations are stacked up and the yield of the properly folded protein decreases with the increase of the number of mutations. After an initial sharp drop, activity is decreasing much slower for the mutants than it is decreasing for the WT enzyme. If we consider the first hour at 90 °C as ‘pre-incubation’ and take activities after 1 hour as 100% for each protein, the retained activity graphs would show the improved stability of the mutants.

[0022] FIG. 12 depicts activity retained after SazCA WT and mutants 2, 3, and 2+3 combination incubation at 90 °C when activity after 1 hour of ‘pre-incubation’ is taken as 100%.

[0023] FIG. 13 depicts activity retained after SspCA WT and mutants incubation at 90 °C. The proteins were induced and expressed at 35 °C and 45 °C temperatures as in Experiment 2.

Protein containing broths were concentrated to boost the initial absolute activity numbers. At five hours, mutant samples were the best performing, retaining about 40% of initial activity.

[0024] FIG. 14 depicts SDS-PAGE analysis of CA6FL protein expressed in *B. subtilis* guided by signal peptide of *B. licheniformis* alpha-amylase (SPamyL) at 35 °C. Lanes 1-3 were the secreted proteins collected at 0, 6 and 12 hours after IPTG induction, respectively. The loading amount is 20 µL (i.e. 15 µL supernatant + 5 µL 4x LDS) per well in non-reducing SDS-PAGE. The red box indicated the expression of CA6FL bands.

[0025] FIG. 15 depicts fresh broth activity for PmaCA (CA3) WT and mutants induced at 30 °C, 35 °C, and 45 °C. Activity for the mut1, mut3, and mut23 (combination of mutants 2 and 3) was below the measurable threshold for the set induced at 30 °C.

[0026] FIG. 16 depicts fresh broth activity for LogaCA (CA4) WT and mutants induced at 35 °C and 45 °C. Activity for the mut3 and mut23 (combination of mutants 2 and 3) was below the measurable threshold for the set induced at 35 °C.

[0027] FIG. 17 depicts fresh broth activity for SspCA (CA5) WT and mutants induced at 35 °C and 45 °C.

[0028] FIG. 18 depicts fresh broth activity for SazCA (CA6) WT and mutants induced at 35 °C and 45 °C.

[0029] FIG. 19 depicts fresh broth activity for PmaCA (CA3) WT and mutants induced at 45 °C with and without addition of cysteine. Activity for the mut1 was below the measurable threshold for the set induced with or without cysteine. Activity for the mut23 (combination of mutants 2 and 3) was below the measurable threshold for the set induced without cysteine.

[0030] FIG. 20 depicts fresh broth activity for PmaCA (CA3) WT and mutants induced at 45 °C with and without addition of cysteine, amino acid mix, or both. Activity for the mut23 (combination of mutants 2 and 3) was below the measurable threshold for the set induced without cysteine.

[0031] FIG. 21 depicts fresh broth activity for PmaCA (CA3) WT and mutants induced at 45 °C with and without addition of diamide. Activity for the mut1, and mut23 (combination of mutants 2 and 3) was below the measurable threshold.

DETAILED DESCRIPTION

[0032] Being one of the fastest enzymes known in nature, carbonic anhydrase (CA) catalyzes the interconversion between CO₂ and bicarbonate which accelerates the capture of CO₂ by

serving as a catalyst in alkaline capture solvents with slow absorption kinetics. The enzyme accelerated process allows use of more benign and sustainable solvents with low regeneration energy thus reducing energy consumption.

[0033] Disclosed herein are CA enzyme candidates with improved catalytic activity, thermostability and solvent compatibility and developed new enzyme immobilization techniques for improving the enzyme longevity and tested more benign and sustainable solvents accelerated by CA for CO₂ capture. The improved enzyme properties together with the novel immobilization technology with selected solvents have the potential to significantly reduce the cost and the energy requirement for CO₂ capture.

[0034] Disclosed herein are optimized, highly active and thermostable carbonic anhydrase enzymes, which are needed for testing in a novel and low energy CO₂ scrubbing process. CA is gaining credibility as an efficient catalyst for significantly enhancing reactive CO₂ absorption in low energy solvents. To overcome the high energy requirement of traditional monoethanolamine (MEA)-based CO₂ scrubbing process, disclosed herein are methods, compositions and systems used to develop more efficient CO₂ scrubbing technology by: 1) improving the robustness of CA, including tolerance to high temperature, high solvent concentration and high pH; 2) improving CA longevity using biodegradable enzyme-entrapping polymeric structures (BEEPS); and 3) utilizing environmentally friendly solvents to improve process sustainability.

[0035] The most studied CA family currently is alpha-class of CAs with at least five members of the family being characterized biochemically and structurally:

[0036] a) *Thermovibrio ammonificans* – TaCA, CA1

[0037] b) *Persephonella marina* EX-H1 – PmaCA, CA3

[0038] c) another *Persephonella marina* CA coming from metagenome sampling at Logachev deep sea vent – LogaCA, CA4

[0039] d) *Sulfurihydrogenibium yellowstonense* YO3AOP1 -SspCA, CA5

[0040] e) *Sulfurihydrogenibium azorense* – SazCA, CA6

[0041] While active site organization of the listed above alpha-class CAs is suited for an independent monomeric function, it seems that all examples (except for TaCA) exist as dimers in the solution, see FIG. 1, for example.

[0042] In, for example FIG.1, the dimerization interface has significant area and is stabilized by hydrophobic interactions, hydrogen bonds and salt bridges. There are no covalent bonds between protein molecules on the dimerization interface. The protein fold is conserved throughout the family and the dimerization interface shares very high similarity among the listed enzymes.

[0043] In an embodiment, disclosed herein are novel protein dimers of alpha-CAs via one or more covalent disulfide bonds designed at the dimerization interface via one or more single-point mutations, replacing a native amino acid residue of the enzyme with cysteine. The exact locations of the single-point mutation may be used in alpha-CAs from different species. Three locations for the intermolecular disulfides were designed in the first round, in an embodiment, PmaCA numbering (including signal peptide, SP) is reflected in FIG. 7.

[0044] In an embodiment, mutants 1, 2, 3, and 2+3 combination were introduced in PmaCA and 2, 3, and 2+3 in SazCA. For SazCA mutant 1 is Gly210Cys + Asn246Cys (numbering according to the full-length sequence including signal peptide), mutant 2 is Ala71Cys, mutant 3 is Ser199Cys + Ser247Cys. Wild-type (WT) enzymes along with the mutants were expressed in *Bacillus subtilis* and induced at 35 °C and 45 °C temperatures. Culturing media containing

secreted enzymes (broth) was collected, cells were spun down and removed. All enzymes were subjected to the prolonged incubation at 90 °C in form of the broth. Samples were taken out at 30 min, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 24 hours, 44-48 hours, and 92 hours. Samples were immediately cooled down to 0 °C and the enzyme activity was tested by Wilbur-Anderson method using colorimetric assay.

[0045] Assay description: In an embodiment, an assay is performed on ice at 0 °C -1 °C temperature. All solutions are chilled on ice until the desired temperature is reached. 1 mL of the 20mM Tris buffer at pH 8.3 was mixed with 0.1mL pH indicator Bromthymol Blue (BTB). Ten μ L (0.01 mL) of broth containing enzyme was added to the mix (nothing added for the control). Then, 1 mL of water fully saturated with CO₂ was added and the stopwatch was started. When BTB changed color from blue to yellow indicating pH dropping below 6.3, stopwatch was stopped. Uncatalyzed reaction time (T_o) is longer than catalyzed reaction time (T_c) when an activity catalyst is present. Activity in Wilbur-Anderson units is calculated as $WAU = (T_o - T_c) / T_c$.

[0046] For the comparison of different enzymes broth activity, this WAU value is then normalized for the dilution factor ($DF = V_{tot} / V_{broth}$ where V_{tot} is a total reaction volume and V_{broth} is the volume of broth added) and optical density of the broth (OD), so the units to compare would be $WAU * DF / OD$.

[0047] For the measurement of retained activity, WAU value of each enzyme at start is taken as 100% for that particular enzyme, and WAU values obtained after various incubation times are compared to the initial WAU activity value.

[0048] As an example, results for PmaCA enzymes set are depicted in FIG. 8.

[0049] To be effective for CO₂ sequestration, CA enzymes need to withstand harsh process conditions, high temperature, high pH, high solvent conditions and tolerance of gas and process

contaminants. In an embodiment, the non-naturally occurring CA enzymes disclosed herein 1) improve enzyme robustness including thermotolerance of CA enzymes with fast CO₂ absorption rate, thermostability and solvent compatibility; 2) improve CA longevity using biodegradable enzyme-entrapping polymeric structures (BEEPS); and 3) utilize compatible environmentally friendly solvents to improve process sustainability with lower energy requirement. Thus, disclosed herein are engineered, non-naturally occurring CA enzymes with improved properties including catalytic activity, thermostability and solvent compatibility.

[0050] In an embodiment, a large quantity of the improved CA enzyme candidates is needed for fabricating sufficient immobilized biocatalyst materials using enzyme immobilization technology and further testing at the bench-scale integrated carbon capture unit with selected more benign and sustainable solvents with low regeneration energy. In an embodiment, the system (with an internal diameter of 7.6 cm, a packing height of approximately 2 m) was outfitted with instrumentation to allow comprehensive data gathering on temperature profile along the absorber and stripper column to calculate mass transfer flux and regeneration energy consumption, optimize the enzyme production process for scaling-up the production of the improved CA enzyme candidates; and produce up to 100 g of protein for fabricating immobilized biocatalyst and testing at the integrated carbon capture unit. The improved enzyme properties together with the novel immobilization technology with selected solvents provide substantial reduction of the energy requirement and cost for CO₂ capture. In an embodiment, the compositions, methods and systems disclosed herein provide alternative CO₂ capture technologies which can be deployed in many industrial applications for capturing CO₂ from biopower and fossil-based power plants.

[0051] The data graphically depicted in FIG. 13 are also disclosed in Table 1 below:

Time, h	CA5 WT 35C	CA5 m2 35C	CA5 m3 35C	CA5 m23 35C	CA5 WT 45C	CA5 m2 45C	CA5 m3 45C	CA5 m23 45C
0	100	100	100	100	100	100	100	100
3	21.1	53.8	56.8	20.6	30.2	37.9	53.3	23.0
5	0.8	8.5	38.5	8.3	0.0	4.4	39.2	13.9

[0052] The data depicted in FIG. 8 are also disclosed in Table 2 below:

CA sample	Activity retained after 2hours at 60C
CA3 WT	14.61
CA3mut1	0.00
CA3mut2	96.24
CA3mut3	95.32

[0053] The data depicted in FIG. 9 are also disclosed in Table 3 below:

time, h	35C WT	35C m1	35C m2	35C m3	35C m23	45C WT	45C m1	45C m2	45C m3	45C m23
0	100	100	100	100	100	100	100	100	100	100
0.5	42.50	10.08	49.41	11.78	0.00	75.11	0.00	76.70	16.92	0.00
1	16.89	0	32.91	0		40.28		50.40884	0.00	
2	11.89		36.11			21.24		48.00		
3	0.00		27.25			5.48		45.31		
4			27.05			3.04		34.39		
6			22.77			0.00		34.80		
48			21.95					24.72		

[0054] The data depicted in FIG. 10 are also disclosed in Table 4 below:

Time, h	CA3 WT 35C	CA3 m1 35C	CA3 m2 35C	CA3 m3 35C	CA3 m23 35C	CA3 WT 45C	CA3 m1 45C	CA3 m2 45C	CA3 m3 45C	CA3 m23 45C
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
0.5	66.70	6.26	55.26	6.09	0.00	44.08	1.14	57.99	5.37	0.00
1	37.24	0.00	42.48	0.00		11.79	0.00	49.40	0.00	
2	18.38		42.42			6.05		43.92		
3	16.30		41.67			3.94		41.39		
4	12.93		37.84			4.52		40.45		
5	15.35		38.74			2.93		43.06		
6	18.18		34.87			0.00		42.14		
24	13.85		31.56			1.09		30.51		
48	6.98		22.80			0.70		23.75		
72	1.89		16.64			0.94		18.85		

[0055] Experimental Examples:

[0056] *B. subtilis* strain and the preparation of competent cells

[0057] *B. subtilis* strain WB800N strain was obtained from MoBiTec GmbH (Gottingen, Germany), and used as the host strain for extracellular expression of CAs. WB800N strain was an eightfold extracellular protease deficient derivative of strain 168, with genotype of nprE aprE epr bpr mpr::ble nprB::bsr Δvpr wprA::hyg cm::neo; NeoR (i.e. carries resistance to neomycin). The competent cells of WB800N were prepared according to the technical guide provided by the above company.

[0058] Expression vector, the design of the constructs for expressing CAs in *B. subtilis*

[0059] *Bacillus* expression vector pHT43 was obtained from MoBiTec GmbH (Gottingen, Germany).

[0060] Signal peptides, gene synthesis and subcloning into vector to build the constructs for expressing CAs in *B. subtilis*

[0061] The signal peptide of *Bacillus licheniformis* alpha-amylase (i.e. AmyL; uniprot ID, P06278) is a 29 aa signal peptide named as SPamyL,

MKQQKRLYARLLTLLFALIFLLPHSAAAA (SEQ ID NO: 35); this signal peptide was used for the expression and secretion of CAs.

[0062] The sequence of each CA gene was codon-optimized using *B. subtilis* codon usage frequency and synthesized by GenScript Inc (Piscataway, New Jersey); it had KpnI site at 5' end, and stop codon-XbaI (taatctaga) at 3' end, and was composed of 87 nucleotides coding for the 29 aa of signal peptide SPamyL, followed by the codon-optimized CA gene sequence, as disclosed herein in SEQ ID NOs 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, and 34.

[0063] For the subcloning, digest the above synthesized gene with KpnI-XbaI, and linked into KpnI-XbaI cut pHT43 vector. The obtained plasmids were used for transformation as described below.

[0064] Transformation and engineered strains

[0065] These above plasmids plus the empty vector pTH43 were transformed into *B. subtilis* WB800N competent cells, using the procedure according to the technical guide provided by the above company. The obtained strains were listed in the below table.

[0066] Table 5. Plasmids and strains for expressing carbonic anhydrase (CA) enzymes in *B. subtilis* using WB800N (shorten as strain 800) as host cell.

Strains	Plasmids with signal peptide and CA description	Amino Acid SEQ ID NO.	DNA SEQ ID NO.
800-1-EV	pHT43 EV		
800-CA3	pHT43-SPamyL-PmaCA-CA3	SEQ ID NO: 1	SEQ ID NO: 2
800-CA3mut1	pHT43-SPamyL-PmaCA-CA3mut1 with G181C and N217C	SEQ ID NO: 3	SEQ ID NO: 4
800-CA3mut2	pHT43-SPamyL-PmaCA-CA3mut2 with A42C	SEQ ID NO: 5	SEQ ID NO: 6
800-CA3mut3	pHT43-SPamyL-PmaCA-CA3mut3 with S170C and N217C	SEQ ID NO: 7	SEQ ID NO: 8
800-CA3mut23	pHT43-SPamyL-PmaCA-CA3mut23	SEQ ID NO: 9	SEQ ID NO: 10
800-CA4	pHT43-SPamyL-LOGACA-CA4	SEQ ID NO: 11	SEQ ID NO: 12

800-CA4mut2	pHT43-SPamyL-LOGACA-CA4mut2	SEQ ID NO: 13	SEQ ID NO: 14
800-CA4mut3	pHT43-SPamyL-LOGACA-CA4mut3	SEQ ID NO: 15	SEQ ID NO: 16
800-CA4mut23	pHT43-SPamyL-LOGACA-CA4mut23	SEQ ID NO: 17	SEQ ID NO: 18
800-CA5	pHT43-SPamyL-SspCA-CA5	SEQ ID NO: 19	SEQ ID NO: 20
800-CA5mut2	pHT43-SPamyL-SspCA-CA5mut2	SEQ ID NO: 21	SEQ ID NO: 22
800-CA5mut3	pHT43-SPamyL-SspCA-CA5mut3	SEQ ID NO: 23	SEQ ID NO: 24
800-CA5mut23	pHT43-SPamyL-SspCA-CA5mut23	SEQ ID NO: 25	SEQ ID NO: 26
800-CA6FL	pHT43-SPamyL-fullSazCA	SEQ ID NO: 27	SEQ ID NO: 28
800-CA6FLmut2	pHT43-SPamyL-fullSazCAmut2	SEQ ID NO: 29	SEQ ID NO: 30
800-CA6FLmut3	pHT43-SPamyL-fullSazCAmut3	SEQ ID NO: 31	SEQ ID NO: 32
800-CA6FLmut23	pHT43-SPamyL-fullSazCAmut23	SEQ ID NO: 33	SEQ ID NO: 34

[0067] Expression and secretion of CA proteins induced with IPTG at the default 35 °C

[0068] Since the plasmids we built contain signal peptide SPamyL, the recombinant CAs were expected to be secreted into the medium. To test the secretion of CAs, obtained transformants were cultured. Briefly, inoculate the recombinant *B. subtilis* strains from plate or glycerol storage into 5 mL fresh 2xYT medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, final pH 7.0) supplemented with neomycin 10 µg/mL and chloramphenicol (5 µg/mL), and cultured in a shaker at 35 °C, 210 rpm. Inoculated the above seed culture into 20 mL fresh 2xYT medium supplemented with neomycin 10 µg/mL and chloramphenicol (5 µg/mL) in a 125-mL flask to an OD₆₀₀ of 0.15. The cultures were grown in a shaker at 35°C, 210 rpm until the OD₆₀₀ reached 0.7 – 0.8, then it was induced with 1 mM IPTG and 0.5 mM ZnSO₄, by which two aliquots of samples being collected and defined as T₀: 100 uL and 1 mL). The cultures were continued to grow in a shaker at 35 °C and 130 rpm.

[0069] Similarly, three aliquots were collected at 6 and 12 h after the induction (defined as T₆ and T₁₂ samples): 100 µL and 1 mL. These T₀ and T₁₂ samples were centrifuged at 12,000 rpm, 10 min, 4 °C to separate the supernatants and pellets. While 100 µL supernatant was mixed with 33 µL 4 x LDS sample buffer, the pellets from 100 uL culture were suspended in 133 µL 1x LDS sample buffer; both being heated at 95 °C for 5 min, followed by centrifugation at 12,000 rpm for 2 min to remove any debris. For these protein samples, 20 µL of each preparation was analyzed with SDS-PAGE.

[0070] Expression and secretion of CA proteins induced with IPTG at 30, 35 and 45 °C

[0071] The expression and secretion of CA proteins by the mutants were also examined after being induced at 30, 35 and 45 °C for 12 hours. The procedures for seed culture preparation, the inoculation into the fresh 2XYT and the initial culturing to OD₆₀₀ of 0.7-0.8 at the default 35 °C were the same as described in the above section of “Expression and secretion of CA proteins induced at the default 35 °C”. When the OD₆₀₀ reached 0.7 – 0.8, 1 mM IPTG and 0.5 mM ZnSO₄ (final concentration) were added into 20 mL culture in 125-mL flasks. The flasks were transferred to different shakers set at designated either 30 °C, or 35 °C, or 45 °C, with a speed of 130 rpm for 12 hours. The samples were harvested and centrifuged as described above, with the supernatants being collected and stored at 4 °C until being analyzed for the CA activity, heat treatment and thermostability analyses.

[0072] Expression and secretion of CA proteins induced with IPTG with the supplements of cysteine or diamide

[0073] To test if the supplements of cysteine or diamide has any impacts on the folding, secretion and functionality of CA proteins, the expression and secretion of CA proteins by the mutants were investigated after being induced at OD₆₀₀ of 0.7-0.8 with a mixture of IPTG and

ZnSO₄ without (as the control) or with cysteine or diamide, using the final concentrations as listed below, followed by continuing shaking at 130 rpm at designated 30 °C, or 35 °C, or 45 °C for 12 hours.

[0074] Treatments with cysteine or diamide supplements (with final concentration added into the medium at OD₆₀₀ of 0.7-0.8):

[0075] (1). Control treatment: 1 mM IPTG + 0.5 mM ZnSO₄

[0076] (2). Cysteine treatment: 1 mM IPTG + 0.5 mM ZnSO₄ + 4 mM cysteine

[0077] (3). Diamide treatment: 1 mM IPTG + 0.5 mM ZnSO₄ + 0.25 mM diamide

[0078] By default, the cysteine stock was freshly prepared unless it was indicated otherwise. The diamide stock also was also freshly prepared.

[0079] **Expression and secretion of CA6FL as a representative CA**

[0080] SDS-PAGE analysis of the cell supernatants reveal that we have successfully expressed CA6FL as a representative CA with signal peptide SPamyL. Furthermore, 6 h of IPTG induction at 35 °C is sufficient to lead CA6FL expression and secretion at substantial levels, while a longer IPTG induction time to 12 hours led to higher expression and secretion levels of CA6FL (FIG. 14). Thus, 12 h of IPTG induction at 35 °C was more desirable than 6 h IPTG induction.

[0081] In an embodiment, elevated temperature leads to better CA expression in *B. subtilis*.

Experiment 1: Wild-type (WT) enzymes along with the mutants were expressed in *Bacillus subtilis* and induced at 30 °C, 35 °C, and 45 °C for PmaCA (CA3), see FIG. 15.

[0082] Experiment 2: Wild-type (WT) enzymes along with the mutants were expressed in *Bacillus subtilis* and induced at 35 °C, and 45 °C for LogaCA, see FIG. 16.

[0083] Experiment 3: Wild-type (WT) enzymes along with the mutants were expressed in *Bacillus subtilis* and induced at 35 °C, and 45 °C for SspCA (CA5), see FIG. 17.

[0084] Experiment 4: Wild-type (WT) enzymes along with the mutants were expressed in *Bacillus subtilis* and induced at 35 °C, and 45 °C for SazCA (CA6), see FIG. 18.

[0085] In an embodiment, addition of free cysteine to the expression media leads to the better CA expression. Experiment 5: Wild-type (WT) enzymes along with the mutants were expressed in *Bacillus subtilis* and induced at 45 °C for PmaCA (CA3) with and without addition of the cysteine, see FIG. 19.

[0086] Experiment 6: Wild-type (WT) enzymes along with the mutants were expressed in *Bacillus subtilis* and induced at 45 °C for PmaCA (CA3) with and without addition of the cysteine, see FIG. 20.

[0087] In an embodiment, addition of diamide to the expression media leads to the improved CA expression. Experiment 7: Wild-type (WT) enzymes along with the mutants were expressed in *Bacillus subtilis* and induced at 45 °C for PmaCA (CA3) with and without addition of the diamide, see FIG. 21.

[0088] The following sequences are embodiments of amino acid and nucleotide sequences representing the genes encoding for engineered CAs disclosed herein.

[0089] SEQ ID NO:1 and SEQ ID NO: 2

[0090] SEQ name: SPamyL-PmaCA-CA3

[0091] LENGTH: 253 for PRT; 762 for DNA

[0092] TYPE: PRT; DNA

[0093] ORGANISM: Signal peptide SPamyL from *Bacillus licheniformis*; PmaCA-CA3 PRT from *Persephonella marina*. Synthetic for DNA

[0094] SEQ ID NO:1 (PRT)

[0095] MKQQKRLYARLLTLLFALIFLLPHSAAAAGGGWSYHGEHGPEHWGDLKDEYI
MCKIGKNQSPVDINRIVDAKLKPIKIEYRAGATKVLNNGHTIKVSYEPGSYIVVDGIKFEL

KQFHFHAPSEHKLKGQHYPFEAHFVHADKHGNNLAVIGVFFKEGRENPILEKIWKVMPEN
AGEEVKLAHKINAEDLLPKDRDYRYSGSLTTPPCSEGVRWIVMEEEMEMSKEQIEKFR
KIMGGDTNRPVQPLNARMIMEK

[0096] SEQ ID NO: 2 (DNA)

[0097] ATGAAACAACAGAAAAGACTGTATGCACGCCTGCTTACATTACTGTTTGCT
CTTATTTTTCTTTTACCGCATTTCAGCAGCGGCTGCCGGCGGAGGATGGAGCTATCAT
GGCGAACATGGACCTGAACATTGGGGTGACCTGAAAGACGAATATATTATGTGCAA
AATCGGCAAAAATCAATCACCGGTTGATATTAACAGAATCGTGGATGCAAACTTA
AACCGATCAAAAATCGAATATCGCGCAGGAGCGACAAAAGTCCTGAACAACGGCCAT
ACAATCAAAGTTTCTTATGAACCGGGATCATATATTGTTGTGGATGGCATCAAATTT
GAATTAAAACAATTTTCATTTTCATGCACCGAGCGAACATAAACTGAAAGGACAGCA
TTATCCGTTTGAAGCTCATTTTGTTCATGCCGATAAACATGGCAATCTGGCTGTCATC
GGAGTTTTCTTTAAAGAAGGCAGAGAAAACCCGATTCTTGAAAAAATCTGGAAAGT
GATGCCGGAAAATGCCGGCGAAGAAGTCAAATTAGCACATAAAATCAACGCGGAA
GATTTACTGCCGAAAGATAGAGATTATTATCGCTATTCAGGAAGCCTGACAACACCG
CCGTGCAGCGAAGGCGTGAGATGGATCGTCATGGAAGAAGAAATGGAAATGTCTAA
AGAACAGATCGAAAAATTTTCGCAAAATCATGGGAGGCGATACGAACCGTCCTGTGC
AGCCGTTGAATGCGAGAATGATTATGGAAAAATAA

[0098] SEQ ID NO:3 and SEQ ID NO: 4

[0099] SEQ name: SPamyL-PmaCA-CA3mut1

[00100] LENGTH: 253 for PRT; 762 for DNA

[00101] TYPE: PRT; DNA

[00102] ORGANISM: Signal peptide SPamyL from *Bacillus licheniformis*; PmaCA-CA3mut1
modified from *Persephonella marina*.

[00103] SEQ ID NO:3 (PRT)

[00104] MKQQKRLYARLLTLLFALIFLLPHSAAAAGGGWSYHGEHGPEHWGDLKDEYI
MCKIGKNQSPVDINRIVDAKLKPIKIEYRAGATKVLNNGHTIKVSYEPGSYIVVDGIKFUL
KQFHFHAPSEHKLKGQHYPFEAHFVHADKHGNNLAVIGVFFKEGRENPILEKIWKVMPEN

AGEEVKLAHKINAEDLLPKDRDYRYSGSLTPPCSECVRWIVMEEEMEMSKEQIEKFR
KIMGGDTNRPVQPLCARMIMEK

[00105] SEQ ID NO: 4 (DNA)

[00106] ATGAAACAACAGAAAAGACTGTATGCACGCCTGCTTACATTACTGTTTGCT
CTTATTTTTCTTTTACCGCATTTCAGCAGCGGCTGCCGGCGGAGGATGGAGCTATCAT
GGCGAACATGGACCTGAACATTGGGGTGACCTGAAAGACGAATATATTATGTGCAA
AATCGGCAAAAATCAATCACCGGTTGATATTAACAGAATCGTGGATGCAAACTTA
AACCGATCAAAATCGAATATCGCGCAGGAGCGACAAAAGTCCTGAACAACGGCCAT
ACAATCAAAGTTTCTTATGAACCGGGATCATATATTGTTGTGGATGGCATCAAATTT
GAATTAACAATTTTCATTTTCATGCACCGAGCGAACATAAACTGAAAGGACAGCA
TTATCCGTTTGAAGCTCATTTTGTTCATGCCGATAAACATGGCAATCTGGCTGTCATC
GGAGTTTTCTTTAAAGAAGGCAGAGAAAACCCGATTCTTGAAAAAATCTGGAAAGT
GATGCCGGAAAATGCCGGCGAAGAAGTCAAATTAGCACATAAAATCAACGCGGAA
GATTTACTGCCGAAAGATAGAGATTATTATCGCTATTCAGGAAGCCTGACAACACCG
CCGTGCAGCGAATGCGTGAGATGGATCGTCATGGAAGAAGAAATGGAAATGTCTAA
AGAACAGATCGAAAAATTTTCGCAAAATCATGGGAGGCGATACGAACCGTCCTGTGC
AGCCGTTGTGTGCGAGAATGATTATGGAAAAATAA

[00107] SEQ ID NO: 5 and SEQ ID NO: 6

[00108] SEQ name: SPamyL-PmaCA-CA3mut2

[00109] LENGTH: 253 for PRT; 762 for DNA

[00110] TYPE: PRT; DNA

[00111] ORGANISM: Signal peptide SPamyL from *Bacillus licheniformis*; PmaCA-CA3mut2
modified from *Persephonella marina*.

[00112] SEQ ID NO: 5 (PRT)

[00113] MKQQKRLYARLLTLLFALIFLLPHSAAAAGGGWSYHGEHGPEHWGDLKDEYI
MCKIGKNQSPVDINRIVDCKLKPIKIEYRAGATKVLNNGHTIKVSYEPGSYIVVDGIKFEL
KQFHFHAPSEHKLGQHYPFEAHFVHADKHGNLAVIGVFFKEGRENPILEKIWKVMPEN

AGEEVKLAHKINAEDLLPKDRDYRYSGSLTPPCSEGVVRWIVMEEEMEMSKEQIEKFR
KIMGGDTNRPVQPLNARMIMEK

[00114] SEQ ID NO: 6 (DNA)

[00115] ATGAAACAACAGAAAAGACTGTATGCACGCCTGCTTACATTACTGTTTGCT
CTTATTTTTCTTTTACCGCATTTCAGCAGCGGCTGCCGGCGGAGGATGGAGCTATCAT
GGCGAACATGGACCTGAACATTGGGGTGACCTGAAAGACGAATATATTATGTGCAA
AATCGGCAAAAATCAATCACCGGTTGATATTAACAGAATCGTGGATTGTAACTTAA
ACCGATCAAAATCGAATATCGCGCAGGAGCGACAAAAGTCCTGAACAACGGCCATA
CAATCAAAGTTTCTTATGAACCGGGATCATATATTGTTGTGGATGGCATCAAATTTG
AATTAAAACAATTTTCATTTTCATGCACCGAGCGAACATAAACTGAAAGGACAGCATT
ATCCGTTTGAAGCTCATTTTGTTCATGCCGATAAACATGGCAATCTGGCTGTCATCG
GAGTTTTCTTTAAAGAAGGCAGAGAAAACCCGATTCTTGAAAAAATCTGGAAAGTG
ATGCCGGAAAATGCCGGCGAAGAAGTCAAATTAGCACATAAAATCAACGCGGAAG
ATTTACTGCCGAAAGATAGAGATTATTATCGCTATTCAGGAAGCCTGACAACACCGC
CGTGCAGCGAAGGCGTGAGATGGATCGTCATGGAAGAAGAAATGGAAATGTCTAAA
GAACAGATCGAAAAATTTTCGCAAAATCATGGGAGGCGATACGAACCGTCCTGTGCA
GCCGTTGAATGCGAGAATGATTATGGAAAAATAA

[00116] SEQ ID NO: 7 and SEQ ID NO: 8

[00117] SEQ name: SPamyL-PmaCA-CA3mut3

[00118] LENGTH: 253 for PRT; 762 for DNA

[00119] TYPE: PRT; DNA

[00120] ORGANISM: Signal peptide SPamyL from *Bacillus licheniformis*; PmaCA-CA3mut3
modified from *Persephonella marina*.

[00121] SEQ ID NO: 7 (PRT)

[00122] MKQQKRLYARLLTLLFALIFLLPHSAAAAGGGWSYHGEHGPEHWGDLKDEYI
MCKIGKNQSPVDINRIVDAKLKPIKIEYRAGATKVLNNGHTIKVSYEPGSYIVVDGIKFEL
KQFHFHAPSEHKLGQHYPFEAHFVHADKHGNLAVIGVFFKEGRENPILEKIWKVMPEN

AGEEVKLAHKINAEDLLPKDRDYRYCGSLTTPPCSEGVVRWIVMEEEMEMSKEQIEKFR
KIMGGDTNRPVQPLCARMIMEK

[00123] SEQ ID NO: 8 (DNA)

[00124] ATGAAACAACAGAAAAGACTGTATGCACGCCTGCTTACATTACTGTTTGCT
CTTATTTTTCTTTTACCGCATTTCAGCAGCGGCTGCCGGCGGAGGATGGAGCTATCAT
GGCGAACATGGACCTGAACATTGGGGTGACCTGAAAGACGAATATATTATGTGCAA
AATCGGCAAAAATCAATCACCGGTTGATATTAACAGAATCGTGGATGCAAACTTA
AACCGATCAAAATCGAATATCGCGCAGGAGCGACAAAAGTCCTGAACAACGGCCAT
ACAATCAAAGTTTCTTATGAACCGGGATCATATATTGTTGTGGATGGCATCAAATTT
GAATTAACAATTTTCATTTTCATGCACCGAGCGAACATAAACTGAAAGGACAGCA
TTATCCGTTTGAAGCTCATTTTGTTCATGCCGATAAACATGGCAATCTGGCTGTCATC
GGAGTTTTCTTTAAAGAAGGCAGAGAAAACCCGATTCTTGAAAAAATCTGGAAAGT
GATGCCGGAAAATGCCGGCGAAGAAGTCAAATTAGCACATAAAATCAACGCGGAA
GATTTACTGCCGAAAGATAGAGATTATTATCGCTATTGTGGAAGCCTGACAACACCG
CCGTGCAGCGAAGGCGTGAGATGGATCGTCATGGAAGAAGAAATGGAAATGTCTAA
AGAACAGATCGAAAAATTTTCGCAAAATCATGGGAGGCGATACGAACCGTCCTGTGC
AGCCGTTGTGTGCGAGAATGATTATGGAAAAATAA

[00125] SEQ ID NO: 9 and SEQ ID NO: 10

[00126] SEQ name: SPamyL-PmaCA-CA3mut23

[00127] LENGTH: 253 for PRT; 762 for DNA

[00128] TYPE: PRT; DNA

[00129] ORGANISM: Signal peptide SPamyL from *Bacillus licheniformis*; PmaCA-CA3mut23
modified from *Persephonella marina*.

[00130] SEQ ID NO: 9 (PRT)

[00131] MKQQKRLYARLLTLLFALIFLLPHSAAAAGGGWSYHGEHGPEHWGDLKDEYI
MCKIGKNQSPVDINRIVDCKLKPIKIEYRAGATKVLNNGHTIKVSYEPGSYIVVDGIKFEL
KQFHFHAPSEHKLGQHYPFEAHFVHADKHGNLAVIGVFFKEGRENPILEKIWKVMPEN

AGEEVKLAHKINAEDLLPKDRDYRYCGSLTTPPCSEGVVRWIVMEEEMEMSKEQIEKFR
KIMGGDTNRPVQPLNCRMIMEK

[00132] SEQ ID NO: 10 (DNA)

[00133] ATGAAACAACAGAAAAGACTGTATGCACGCCTGCTTACATTACTGTTTGCT
CTTATTTTTCTTTTACCGCATTTCAGCAGCGGCTGCCGGCGGAGGATGGAGCTATCAT
GGCGAACATGGACCTGAACATTGGGGTGACCTGAAAGACGAATATATTATGTGCAA
AATCGGCAAAAATCAATCACCGGTTGATATTAACAGAATCGTGGATTGTAACTTAA
ACCGATCAAAATCGAATATCGCGCAGGAGCGACAAAAGTCCTGAACAACGGCCATA
CAATCAAAGTTTCTTATGAACCGGGATCATATATTGTTGTGGATGGCATCAAATTTG
AATTAAAACAATTTTCATTTTCATGCACCGAGCGAACATAAACTGAAAGGACAGCATT
ATCCGTTTGAAGCTCATTTTGTTCATGCCGATAAACATGGCAATCTGGCTGTCATCG
GAGTTTTCTTTAAAGAAGGCAGAGAAAACCCGATTCTTGAAAAAATCTGGAAAGTG
ATGCCGGAAAATGCCGGCGAAGAAGTCAAATTAGCACATAAAATCAACGCGGAAG
ATTTACTGCCGAAAGATAGAGATTATTATCGCTATTGTGGAAGCCTGACAACACCGC
CGTGCAGCGAAGGCGTGAGATGGATCGTCATGGAAGAAGAAATGGAAATGTCTAAA
GAACAGATCGAAAAATTCGCAAAATCATGGGAGGCGATACGAACCGTCCTGTGCA
GCCGTTGTGTGCGAGAATGATTATGGAAAAATAA

[00134] SEQ ID NO: 11 and SEQ ID NO: 12

[00135] SEQ name: SPamyL-LOGACA-CA4

[00136] LENGTH: 255 for PRT; 768 for DNA

[00137] ORGANISM: Signal peptide SPamyL from *Bacillus licheniformis*; LOGACA-CA4
from deep sea thermal vent.

[00138] SEQ ID NO: 11 (PRT)

[00139] MKQQKRLYARLLTLLFALIFLLPHSAAAAGGVGHWSYHGETGPQHWGDLKNE
YIMCKIGKNQSPVDISRIVEAELEKIKINYSSGGSSITNNGHTIKVSYEPGSYIIVDGIRFEL
KQFHFHAPSEHTIKGKSYPFEAHFVHADKDGNLAVIGVIFKEGKKNPHEKIWENLPEAG
KTIKLAHKINAYDLLPKKKKYRYSGSLTTPPCSEGVVRWIVMEEEMELSKEQIEKFRKL
MGGDTNRPVQPLNARMIMEMD

[00140] SEQ ID NO: 12 (DNA)

[00141] ATGAAACAACAGAAAAGACTGTATGCACGCCTGCTTACATTACTGTTTGCT
CTTATTTTTCTTTTACCGCATTTCAGCAGCGGCTGCCGGCGGAGTTGGACATTGGTCTT
ATCATGGCGAAACAGGACCGCAACATTGGGGCGATCTGAAAAACGAATACATCATG
TGCAAAATCGGCAAAAACCAGTCACCGGTGGATATTAGCAGAATCGTCGAAGCTGA
ACTTGAAAAAATCAAAATCAACTATTCAAGCGGCGGATCTTCAATCACAAACAACG
GACATACAATCAAAGTTTCTTATGAACCGGGATCATATATTATCGTGGATGGCATT
GCTTTGAATTAAAACAATTTTCATTTTCATGCCCCGAGCGAACATACAATCAAAGGCA
AATCTTATCCGTTTGAAGCACATTTTGTCCATGCGGATAAAGATGGCAATCTGGCAG
TTATTGGAGTGATCTTTAAAGAAGGCAAGAAAAATCCGATCATCGAAAAAATTTGG
GAAAACTTACCGGAAGCGGGCAAAACAATCAAACCTGGCTCATAAAATCAACGCCTA
TGATCTGCTTCCGAAAAAGAAAAAATACTACAGATACAGCGGATCTCTTACAACAC
CGCCGTGTTTCAAGAGGCGTCCGCTGGATTGTTATGGAAGAAGAAATGGAAGTTAGC
AAAGAACAATCGAAAAATTTAGAAAATGATGGGCGGAGATACAAATAGACCGG
TTCAGCCGTTAAACGCTCGCATGATTATGGAAATGGATTAA

[00142] SEQ ID NO: 13 and SEQ ID NO: 14

[00143] SEQ name: SPamyL-LOGACA-CA4mut2

[00144] LENGTH: 255 for PRT; 768 for DNA

[00145] TYPE: PRT; DNA

[00146] ORGANISM: Signal peptide SPamyL from *Bacillus licheniformis*; LOGACA-CA4mut2 modified from deep sea thermal vent.

[00147] SEQ ID NO: 13 (PRT)

[00148] MKQQKRLYARLLTLLFALIFLLPHSAAAAGGVGHWSYHGETGPQHWGDLKNE
YIMCKIGKNQSPVDISRIVECELEKIKINYSSGGSSITNNGHTIKVSYEPGSYIIVDGIRFEL
KQFHFHAPSEHTIKGKSYPFEAHFVHADKDGNLAVIGVIFKEGKKNPIIEKIWENLPEAG
KTIKLAHKINAYDLLPKKKKYRYSGSLTPPCSEGVRWIVMEEEMELSKEQIEKFRKL
MGGDTNRPVQPLNARMIMEMD

[00149] SEQ ID NO: 14 (DNA)

[00150] ATGAAACAACAGAAAAGACTGTATGCACGCCTGCTTACATTACTGTTTGCT
CTTATTTTTCTTTTACCGCATTTCAGCAGCGGCTGCCGGCGGAGTTGGACATTGGTCTT

ATCATGGCGAAACAGGACCGCAACATTGGGGCGATCTGAAAAACGAATACATCATG
TGCAAAATCGGCAAAAACAGTCACCGGTGGATATTAGCAGAATCGTCGAATGTGA
ACTTGAAAAAATCAAAATCAACTATTCAAGCGGCGGATCTTCAATCACAAACAACG
GACATACAATCAAAGTTTCTTATGAACCGGGATCATATATTATCGTGGATGGCATT
GCTTTGAATTAAAACAATTTTCATTTTCATGCCCCGAGCGAACATACAATCAAAGGCA
AATCTTATCCGTTTGAAGCACATTTTGTCCATGCGGATAAAGATGGCAATCTGGCAG
TTATTGGAGTGATCTTTAAAGAAGGCAAGAAAAATCCGATCATCGAAAAAATTTGG
GAAAACCTTACCGGAAGCGGGCAAAACAATCAAACCTGGCTCATAAAATCAACGCCTA
TGATCTGCTTCCGAAAAAGAAAAAATACTACAGATACAGCGGATCTCTTACAACAC
CGCCGTGTTTCAGAAGGCGTCCGCTGGATTGTTATGGAAGAAGAAATGGAAGTTAGC
AAAGAACAAATCGAAAAATTTAGAAAACCTGATGGGCGGAGATACAAATAGACCGG
TTCAGCCGTAAACGCTCGCATGATTATGGAAATGGATTAA

[00151] SEQ ID NO: 15 and SEQ ID NO: 16

[00152] SEQ name: SPamyL-LOGACA-CA4mut3

[00153] LENGTH: 255 for PRT; 768 for DNA

[00154] TYPE: PRT; DNA

[00155] ORGANISM: Signal peptide SPamyL from *Bacillus licheniformis*; LOGACA-CA4mut3 modified from deep sea thermal vent.

[00156] SEQ ID NO: 15 (PRT)

[00157] MKQQKRLYARLLTLLFALIFLLPHSAAAAGGVGHWSYHGETGPQHWGDLKNE
YIMCKIGKNQSPVDISRIVEAELEKIKINYSSGGSSITNNGHTIKVSYEPGSYIIVDGIRFEL
KQFHFHAPSEHTIKGKSYPFEAHFVHADKDGNLAVIGVIFKEGKKNPHEKIWENLPEAG
KTIKLAHKINAYDLLPKKKKYYRYCGSLTPPCSEGVVRWIVMEEEMELSKEQIEKFRKL
MGGDTNRPVQPLNCRMIMEMD

[00158] SEQ ID NO: 16 (DNA)

[00159] ATGAAACAACAGAAAAGACTGTATGCACGCCTGCTTACATTACTGTTTGCT
CTTATTTTTCTTTTACCGCATTTCAGCAGCGGCTGCCGGCGGAGTTGGACATTGGTCTT
ATCATGGCGAAACAGGACCGCAACATTGGGGCGATCTGAAAAACGAATACATCATG
TGCAAAATCGGCAAAAACAGTCACCGGTGGATATTAGCAGAATCGTCGAAGCTGA

ACTTGAAAAAATCAAAATCAACTATTCAAGCGGCGGATCTTCAATCACAAACAACG
GACATACAATCAAAGTTTCTTATGAACCGGGATCATATATTATCGTGGATGGCATT
GCTTTGAATTAAAACAATTTTCATTTTCATGCCCCGAGCGAACATACAATCAAAGGCA
AATCTTATCCGTTTGAAGCACATTTTGTCCATGCGGATAAAGATGGCAATCTGGCAG
TTATTGGAGTGATCTTTAAAGAAGGCAAGAAAAATCCGATCATCGAAAAAATTTGG
GAAAACCTACCGGAAGCGGGCAAAACAATCAAACCTGGCTCATAAAATCAACGCCTA
TGATCTGCTTCCGAAAAAGAAAAAATACTACAGATACTGCGGATCTCTTACAACACC
GCCGTGTTTCAAGGCGTCCGCTGGATTGTTATGGAAGAAGAAATGGAACCTAGCA
AAGAACAATCGAAAAATTTAGAAAACCTGATGGGCGGAGATACAAATAGACCGGTT
CAGCCGTAAACTGTCGCATGATTATGGAAATGGATTAA

[00160] SEQ ID NO: 17 and SEQ ID NO: 18

[00161] SEQ name: SPamyL-LOGACA-CA4mut23

[00162] LENGTH: 255 for PRT; 768 for DNA

[00163] TYPE: PRT; DNA

[00164] ORGANISM: Signal peptide SPamyL from *Bacillus licheniformis*; LOGACA-CA4mut23 modified from deep sea thermal vent.

[00165] SEQ ID NO: 17 (PRT)

[00166] MKQQKRLYARLLTLLFALIFLLPHSAAAAGGVGHWSYHGETGPQHWGDLKNE
YIMCKIGKNQSPVDISRIVECELEKIKINYSSGGSSITNNGHTIKVSYEPGSYIIVDGIRFEL
KQFHFHAPSEHTIKGKSYPFEAHFVHADKDGNLAVIGVIFKEGKKNPHEKIWENLPEAG
KTIKLAHKINAYDLLPKKKKYRYCGSLTPPCSEGVVRWIVMEEEMELSKEQIEKFRKL
MGGDTNRPVQPLNCRMIMEMD

[00167] SEQ ID NO: 18 (DNA)

[00168] ATGAAACAACAGAAAAGACTGTATGCACGCCTGCTTACATTACTGTTTGCT
CTTATTTTTCTTTTACCGCATTTCAGCAGCGGCTGCCGGCGGAGTTGGACATTGGTCTT
ATCATGGCGAAACAGGACCGCAACATTGGGGCGATCTGAAAAACGAATACATCATG
TGCAAAATCGGCAAAAACAGTCACCGGTGGATATTAGCAGAATCGTCGAATGTGA
ACTTGAAAAAATCAAAATCAACTATTCAAGCGGCGGATCTTCAATCACAAACAACG
GACATACAATCAAAGTTTCTTATGAACCGGGATCATATATTATCGTGGATGGCATT

GCTTTGAATTAAAACAATTTTCATTTTCATGCCCCGAGCGAACATACAATCAAAGGCA
AATCTTATCCGTTTGAAGCACATTTTGTCCATGCGGATAAAGATGGCAATCTGGCAG
TTATTGGAGTGATCTTTAAAGAAGGCAAGAAAAATCCGATCATCGAAAAAATTTGG
GAAAACTTACCGGAAGCGGGCAAAACAATCAAACCTGGCTCATAAAATCAACGCCTA
TGATCTGCTTCCGAAAAAGAAAAAATACTACAGATACTGCGGATCTCTTACAACACC
GCCGTGTTTACAAGGCGTCCGCTGGATTGTTATGGAAGAAGAAATGGAACCTTAGCA
AAGAACAATCGAAAAATTTAGAAAATGATGGGCGGAGATACAAATAGACCGGTT
CAGCCGTAAACTGTTCGCATGATTATGGAAATGGATTAA

[00169] SEQ ID NO: 19 and SEQ ID NO: 20

[00170] SEQ name: SPamyL-SspCA-CA5

[00171] LENGTH: 255 for PRT; 768 for DNA

[00172] TYPE: PRT; DNA

[00173] ORGANISM: Signal peptide SPamyL from *Bacillus licheniformis*; SspCA-CA5 from *Sulfurihydrogenibium* sp. strain YO3AOP1.

[00174] SEQ ID NO: 19 (PRT)

[00175] MKQQKRLYARLLTLLFALIFLLPHSAAAAEHEWSYEGEKGPEHWAQLKPEFF
WCKLKNQSPINIDKKYKVKANLPKLNLYYKTAKESVVNNGHTIQINIKEDNTLNYLGE
KYQLKQFHFHTPSEHTIEKKSYPLEIHFVHKTEDGKILVVGVMKLGKTNKELDKILNV
APAEEGEKILDKNLNLNLPKDKRYMTYSGSLTTPPCTEGVRWIVLKKPISISKQQLEKL
KSVMVNPNNRPVQEINSRWIIEGF

[00176] SEQ ID NO: 20 (DNA)

[00177] ATGAAACAACAGAAAAGACTGTATGCACGCCTGCTTACATTACTGTTTGCT
CTTATTTTTCTTTTACCGCATTTCAGCAGCGGCTGCCGAACATGAATGGTCTTATGAAG
GCGAAAAAGGACCGGAACATTGGGCACAACCTGAAACCGGAATTTTTCTGGTGCAAA
CTTAAAAACCAGTCACCGATCAACATCGATAAAAAATACAAAGTTAAAGCTAACCT
GCCGAACTGAACCTTTACTACAAAACAGCCAAAGAATCAGAAGTTGTGAATAACG
GACATACAATCCAAATCAACATCAAAGAAGATAACACACTTAACTACCTGGGCGAA
AAATACCAACTGAAACAGTTTTCATTTTCATACACCGAGCGAACATACAATCGAGAA
AAAATCATACCCGCTTGAAATCCATTTTGTCCATAAAACAGAAGATGGCAAAATCCT

TGTCGTTGGAGTTATGGCTAAACTGGGCAAAACAAACAAAGAATTAGATAAAATTC
TGAACGTGGCACCGGCGGAAGAAGGAGAAAAAATCTTAGATAAAAACCTGAACCTG
AACAACCTGATCCCGAAAGATAAAAGATACATGACATACTCAGGAAGCCTTACAAC
ACCGCCGTGTACAGAAGGCGTTCGCTGGATCGTGCTGAAAAAACCGATCTCTATTTC
AAAACAACAGCTGGAAAACTTAAATCAGTGATGGTCAATCCGAATAACAGACCGG
TCCAGGAAATTAACAGCCGCTGGATTATCGAAGGCTTTTAA

[00178] SEQ ID NO: 21 and SEQ ID NO: 22

[00179] SEQ name: SPamyL-SspCA-CA5mut2

[00180] LENGTH: 255 for PRT; 768 for DNA

[00181] TYPE: PRT; DNA

[00182] ORGANISM: Signal peptide SPamyL from *Bacillus licheniformis*; SspCA-CA5mut2 modified from *Sulfurihydrogenibium* sp. strain YO3AOP1.

[00183] SEQ ID NO: 21 (PRT)

[00184] MKQQKRLYARLLTLLFALIFLLPHSAAAAEHEWSYEGEKGPEHWAQLKPEFF
WCKLKNQSPINIDKKYKVKCNLPKLNLYYKTAKESVVNNGHTIQINIKEDNTLNYLGE
KYQLKQFHFHTPSEHTIEKKSYPLEIHFVHKTEDGKILVVGVMKLGKTNKELDKILNV
APAEERGEKILDKNLNLNLPKDKRYMTYSGSLTTPCTEGVRWIVLKKPISISKQQLEKL
KSVMVNPNNRPVQEINSRWIIIEGF

[00185] SEQ ID NO: 22 (DNA)

[00186] ATGAAACAACAGAAAAGACTGTATGCACGCCTGCTTACATTACTGTTTGCT
CTTATTTTTCTTTTACCGCATTTCAGCAGCGGCTGCCGAACATGAATGGTCTTATGAAG
GCGAAAAAGGACCGGAACATTGGGCACAACTGAAACCGGAATTTTTCTGGTGCAAA
CTTAAAAACCAGTCACCGATCAACATCGATAAAAAATACAAAGTTAAATGTAACCT
GCCGAACTGAACCTTTACTACAAAACAGCCAAAGAATCAGAAGTTGTGAATAACG
GACATACAATCCAAATCAACATCAAAGAAGATAACACACTTAACCTGGGCGAA
AAATACCAACTGAAACAGTTTCATTTTCATACACCGAGCGAACATACAATCGAGAA
AAAATCATACCCGCTTGAAATCCATTTTGTCCATAAAACAGAAGATGGCAAAATCCT
TGTCGTTGGAGTTATGGCTAAACTGGGCAAAACAAACAAAGAATTAGATAAAATTC
TGAACGTGGCACCGGCGGAAGAAGGAGAAAAAATCTTAGATAAAAACCTGAACCTG

AACAACCTGATCCCGAAAGATAAAAAGATACATGACATACTCAGGAAGCCTTACAAC
ACCGCCGTGTACAGAAGGCGTTCGCTGGATCGTGCTGAAAAAACCGATCTCTATTTC
AAAACAACAGCTGGAAAACTTAAATCAGTGATGGTCAATCCGAATAACAGACCGG
TCCAGGAAATTAACAGCCGCTGGATTATCGAAGGCTTTTAA

[00187] SEQ ID NO: 23 and SEQ ID NO: 24

[00188] SEQ name: SPamyL-SspCA-CA5mut3

[00189] LENGTH: 255 for PRT; 768 for DNA

[00190] TYPE: PRT; DNA

[00191] ORGANISM: Signal peptide SPamyL from *Bacillus licheniformis*; SspCA-CA5mut3 modified from *Sulfurihydrogenibium* sp. strain YO3AOP1.

[00192] SEQ ID NO: 23 (PRT)

[00193] MKQQKRLYARLLTLLFALIFLLPHSAAAAEHEWSYEGEKGPEHWAQLKPEFF
WCKLKNQSPINIDKKYKVKANLPKLNLYYKTAKESVNNNGHTIQINIKEDNTLNYLGE
KYQLKQFHFHTPSEHTIEKKSYPLEIHFVHKTEDGKILVGVMAKLGKTNKELDKILNV
APAEEGEKILDKNLNLNNLIPKDKRYMTYCGSLTTPPCTEGVRWIVLKKPISISKQQLEK
LKSVNMVNPNNRPVQEINCRWIIIEGF

[00194] SEQ ID NO: 24 (DNA)

[00195] ATGAAACAACAGAAAAGACTGTATGCACGCCTGCTTACATTACTGTTTGCT
CTTATTTTTCTTTTACCGCATTTCAGCAGCGGCTGCCGAACATGAATGGTCTTATGAAG
GCGAAAAAGGACCGGAACATTGGGCACAACCTGAAACCGGAATTTTTCTGGTGCAAA
CTTAAAAACCAGTCACCGATCAACATCGATAAAAAATACAAAGTTAAAGCTAACCT
GCCGAAACTGAACCTTTACTACAAAACAGCCAAAGAATCAGAAGTTGTGAATAACG
GACATACAATCCAAATCAACATCAAAGAAGATAACACACTTAACTACCTGGGCGAA
AAATACCAACTGAAACAGTTTCATTTTCATACACCGAGCGAACATACAATCGAGAA
AAAATCATACCCGCTTGAAATCCATTTTGTCCATAAAACAGAAGATGGCAAAATCCT
TGTCGTTGGAGTTATGGCTAAACTGGGCAAAACAAACAAAGAATTAGATAAAATTC
TGAACGTGGCACCGGCGGAAGAAGGAGAAAAAATCTTAGATAAAAACCTGAACCTG
AACAACCTGATCCCGAAAGATAAAAAGATACATGACATACTGCGGAAGCCTTACAAC
ACCGCCGTGTACAGAAGGCGTTCGCTGGATCGTGCTGAAAAAACCGATCTCTATTTC

AAAACAACAGCTGGAAAACTTAAATCAGTGATGGTCAATCCGAATAACAGACCGG
TCCAGGAAATTAAGTGTGCTGGATTATCGAAGGCTTTTAA

[00196] SEQ ID NO: 25 and SEQ ID NO: 26

[00197] SEQ name: SPamyL-SspCA-CA5mut23

[00198] LENGTH: 255 for PRT; 768 for DNA

[00199] TYPE: PRT; DNA

[00200] ORGANISM: Signal peptide SPamyL from *Bacillus licheniformis*; SspCA-CA5mut23 modified from *Sulfurihydrogenibium* sp. strain YO3AOP1.

[00201] SEQ ID NO: 25 (PRT)

[00202] MKQKRLYARLLTLLFALIFLLPHSAAAAEHEWSYEGEKGPHEWAQLKPEFF
WCKLKNQSPINIDKKYKVKCNLPKLNLYYKTAKESVNNNGHTIQINIKEDNTLNLYLGE
KYQLKQFHFHTPSEHTIEKKSYPLEIHFVHKTEDGKILVVGVMKLGKTNKELDKILNV
APAEEGEKILDKNLNLNLPKDKRYMTYCGSLTTPPCTEGVRWIVLKKPISISKQQLEK
LKSVMVNPNNRPVQEINCRWIIIEGF

[00203] SEQ ID NO: 26 (DNA)

[00204] ATGAAACAACAGAAAAGACTGTATGCACGCCTGCTTACATTACTGTTTGCT
CTTATTTTTCTTTTACCGCATTTCAGCAGCGGCTGCCGAACATGAATGGTCTTATGAAG
GCGAAAAAGGACCGGAACATTGGGCACAACTGAAACCGGAATTTTTCTGGTGCAAA
CTTAAAAACCAGTCACCGATCAACATCGATAAAAAATACAAAGTTAAATGTAACCT
GCCGAACTGAACCTTTACTACAAAACAGCCAAAGAATCAGAAGTTGTGAATAACG
GACATACAATCCAAATCAACATCAAAGAAGATAACACACTTAACTACCTGGGCGAA
AAATACCAACTGAAACAGTTTCATTTTCATACACCGAGCGAACATACAATCGAGAA
AAAATCATACCCGCTTGAAATCCATTTTGTCCATAAAACAGAAGATGGCAAAATCCT
TGTCGTTGGAGTTATGGCTAAACTGGGCAAAACAAACAAAGAATTAGATAAAATTC
TGAACGTGGCACCGGCGGAAGAAGGAGAAAAAATCTTAGATAAAAACCTGAACCTG
AACAACTGATCCCGAAAGATAAAAGATACATGACATACTGCGGAAGCCTTACAAC
ACCGCCGTGTACAGAAGGCGTTCGCTGGATCGTGCTGAAAAAACCGATCTCTATTTC
AAAACAACAGCTGGAAAACTTAAATCAGTGATGGTCAATCCGAATAACAGACCGG
TCCAGGAAATTAAGTGTGCTGGATTATCGAAGGCTTTTAA

[00205] SEQ ID NO: 27 and SEQ ID NO: 28

[00206] SEQ name: SPamyL-SazCA-CA6FL

[00207] LENGTH: 266 for PRT; 801 for DNA

[00208] TYPE: PRT; DNA

[00209] ORGANISM: Signal peptide SPamyL from *Bacillus licheniformis*; SazCA-CA6FL from *Sulfurihydrogenibium azorense*

[00210] SEQ ID NO: 27 (PRT)

[00211] MKQQKRLYARLLTLLFALIFLLPHSAAAAGEHAILQKNAEVHHWSYEGENGPE
NWAKLNPEYFWCNLKNQSPVDISDNYKVHAKLEKHLHINYNKAVNPEIVNNGHTIQVNV
LEDFKLNIGKEYHLKQFHFHAPSEHTVNGKYYPLEMLVHKDKDGNIAVIGVFFKEG
KANPELDKVFKNALKEEGSKVFDGSININALLPPVKNNYTYSGSLTTPPCTEGVLWIVLK
QPITASKQQIELFKSIMKHNNNRPTQPINSRYILES

[00212] SEQ ID NO: 28 (DNA)

[00213] In an embodiment, also referred to as: >2_Gene6-pH43-SPamyL-fullSazCA

[00214] ATGAAACAACAGAAAAGACTGTATGCACGCCTGCTTACATTACTGTTTGCT
CTTATTTTTCTTTTACCGCATTTCAGCAGCGGCTGCCGGCGAACATGCAATTCTGCAGA
AAAATGCGGAAGTCCATCATTGGAGCTATGAAGGCGAAAACGGACCGGAAAATTGG
GCCAACTGAACCCGGAATACTTTTGGTGCAACCTTAAAAACAGTCTCCGGTCGAT
ATTCAGATAACTACAAAGTTCATGCCAACTGGAAAACTGCATATCAACTACAAC
AAAGCAGTTAACCCGGAATTGTGAATAACGGACATACAATCCAAGTTAACGTGTT
AGAAGATTTTAACTGAACATCAAAGGCAAAGAATACCATCTTAAACAGTTTCATTT
TCATGCTCCGTCTGAACATACAGTGAACGGCAAATATTATCCGCTTGAAATGCATCT
GGTCCATAAAGATAAAGATGGCAACATTGCAGTCATCGGAGTTTTCTTTAAAGAAG
GCAAAGCGAACCCGGAAGTTGATAAAGTTTTTAAAAACGCTCTGAAAGAAGAAGGA
AGCAAAGTGTTTGATGGCTCTATTAACATCAATGCGCTGCTTCCGCCGGTTAAAAAC
TACTACATACTCAGGAAGCTTAACAACACCGCCGTGTACAGAAGGCGTGCTGTG
GATTGTCCTTAAACAACCGATCACAGCTTCTAAACAACAGATTGAACTGTTTAAATC
AATCATGAAACATAACAACAATAGACCGACACAGCCGATTAACCTCACGCTATATCC
TGGAAGCAATTAA

[00215] SEQ ID NO: 29 and SEQ ID NO: 30

[00216] SEQ name: SPamyL-SazCA-CA6FLmut2

[00217] LENGTH: 266 for PRT; 801 for DNA

[00218] TYPE: PRT; DNA

[00219] ORGANISM: Signal peptide SPamyL from *Bacillus licheniformis*; SazCA-CA6FLmut2 modified from *Sulfurihydrogenibium azorense*

[00220] SEQ ID NO: 29 (PRT)

[00221] MKQKRLYARLLTLLFALIFLLPHSAAAAGEHAILQKNAEVHHWSYEGENGPE
NWAKLNPEYFWCNLKNQSPVDISDNYKVHCKLEKLEKLNHYNKAVNPEIVNNGHTIQVNV
LEDFKLNIGKEYHLKQFHFHAPSEHTVNGKYYPLEMLVHKDKDGNIAVIGVFFKEG
KANPELDKVFKNALKEEGSKVFDGSININALLPPVKNNYYTYSGLTTPPCTEGVLWIVLK
QPITASKQQIELFKSIMKHNNNRPTQPINSRYILES

[00222] SEQ ID NO: 30 (DNA)

[00223] ATGAAACAACAGAAAAGACTGTATGCACGCCTGCTTACATTACTGTTTGCT
CTTATTTTTCTTTTACCGCATTTCAGCAGCGGCTGCCGGCGAACATGCAATTCTGCAGA
AAAATGCGGAAGTCCATCATTGGAGCTATGAAGGCGAAAACGGACCGGAAAATTGG
GCCAACTGAACCCGGAATACTTTTGGTGCAACCTTAAAAACCAGTCTCCGGTCGAT
ATTCAGATAACTACAAAGTTCATTGTAACTGGAAAACTGCATATCAACTACAAC
AAAGCAGTTAACCCGGAATTGTGAATAACGGACATACAATCCAAGTTAACGTGTT
AGAAGATTTTAACTGAACATCAAAGGCAAAGAATACCATCTTAAACAGTTTCATT
TCATGCTCCGTCTGAACATACAGTGAACGGCAAATATTATCCGCTTGAAATGCATCT
GGTCCATAAAGATAAAGATGGCAACATTGCAGTCATCGGAGTTTTCTTTAAAGAAG
GCAAAGCGAACCCGGAAGTTGATAAAGTTTTTAAAAACGCTCTGAAAGAAGAAGGA
AGCAAAGTGTTTGATGGCTCTATTAACATCAATGCGCTGCTTCCGCCGGTTAAAAAC
TACTACATACTCAGGAAGCTTAACAACACCGCCGTGTACAGAAGGCGTGCTGTG
GATTGTCCTTAAACAACCGATCACAGCTTCTAAACAACAGATTGAACTGTTTAAATC
AATCATGAAACATAACAACAATAGACCGACACAGCCGATTAACCTACGCTATATCC
TGGAAGCAATTAA

[00224] SEQ ID NO: 31 and SEQ ID NO: 32

[00225] SEQ name: SPamyL-SazCA-CA6FLmut3

[00226] LENGTH: 266 for PRT; 801 for DNA

[00227] TYPE: PRT; DNA

[00228] ORGANISM: Signal peptide SPamyL from *Bacillus licheniformis*; SazCA-CA6FLmut3 modified from *Sulfurihydrogenibium azorense*

[00229] SEQ ID NO: 31 (PRT)

[00230] MKQQKRLYARLLTLLFALIFLLPHSAAAAGEHAILQKNAEVHHWSYEGENGPE
NWAKLNPEYFWCNLKNQSPVDISDNYKVHAKLEKLNHYNKAVNPEIVNNGHTIQVNV
LEDFKLNIGKEYHLKQFHFHAPSEHTVNGKYYPLEMLVHKDKDGNIAVIGVFFKEG
KANPELDKVFKNALKEEGSKVFDGSININALLPPVKNNYYTYCGSLTPPCTEGVLWIVLK
QPITASKQQIELFKSIMKHNNNRPTQPINCRYILES

[00231] SEQ ID NO: 32 (DNA)

[00232] ATGAAACAACAGAAAAGACTGTATGCACGCCTGCTTACATTACTGTTTGCT
CTTATTTTTCTTTTACCGCATTACAGCAGCGGCTGCCGGCGAACATGCAATTCTGCAGA
AAAATGCGGAAGTCCATCATTGGAGCTATGAAGGCGAAAACGGACCGGAAAATTGG
GCCAACTGAACCCGGAATACTTTTGGTGCAACCTTAAAAACCAGTCTCCGGTCGAT
ATTCAGATAACTACAAAGTTCATGCCAACTGGAAAACTGCATATCAACTACAAC
AAAGCAGTTAACCCGGAATTGTGAATAACGGACATACAATCCAAGTTAACGTGTT
AGAAGATTTTAACTGAACATCAAAGGCAAAGAATACCATCTTAAACAGTTTCATTT
TCATGCTCCGTCTGAACATACAGTGAACGGCAAATATTATCCGCTTGAAATGCATCT
GGTCCATAAAGATAAAGATGGCAACATTGCAGTCATCGGAGTTTTCTTTAAAGAAG
GCAAAGCGAACCCGGAACCTTGATAAAGTTTTTAAAAACGCTCTGAAAGAAGAAGGA
AGCAAAGTGTTTGATGGCTCTATTAACATCAATGCGCTGCTTCCGCCGGTTAAAAAC
TACTACATACTGCGGAAGCTTAACAACACCGCCGTGTACAGAAGGCGTGCTGTG
GATTGTCCTTAAACAACCGATCACAGCTTCTAAACAACAGATTGAACTGTTTAAATC
AATCATGAAACATAACAACAATAGACCGACACAGCCGATTAAGTGTGCTATATCCT
GGAAAGCAATTAA

[00233] SEQ ID NO: 33 and SEQ ID NO: 34

[00234] SEQ name: SPamyL-SazCA-CA6FLmut23

[00235] LENGTH: 266 for PRT; 801 for DNA

[00236] TYPE: PRT; DNA

[00237] ORGANISM: Signal peptide SPamyL from *Bacillus licheniformis*; SazCA-CA6FLmut23 modified from *Sulfurihydrogenibium azorense*

[00238] SEQ ID NO: 33 (PRT)

[00239] MKQQKRLYARLLTLLFALIFLLPHSAAAAGEHAILQKNAEVHHWSYEGENGPE
NWAKLNPEYFWCNLKNQSPVDISDNYKVHCKLEKLHINYNKAVNPEIVNNGHTIQVNV
LEDFKLNIGKEYHLKQFHFHAPSEHTVNGKYYPLEMLVHKDKDGNIAVIGVFFKEG
KANPELDKVFKNALKEEGSKVFDGSININALLPPVKNNYYTYCGSLTTPPCTEGVLWIVLK
QPITASKQQIELFKSIMKHNNNRPTQPINCRYILES

[00240] SEQ ID NO: 34 (DNA)

[00241] ATGAAACAACAGAAAAGACTGTATGCACGCCTGCTTACATTACTGTTTGCT
CTTATTTTTCTTTTACCGCATTTCAGCAGCGGCTGCCGGCGAACATGCAATTCTGCAGA
AAAATGCGGAAGTCCATCATTGGAGCTATGAAGGCGAAAACGGACCGGAAAATTGG
GCCAACTGAACCCGGAATACTTTTGGTGCAACCTTAAAAACCAGTCTCCGGTCGAT
ATTCAGATAACTACAAAGTTCATTGTAACTGGAAAACTGCATATCAACTACAAC
AAAGCAGTTAACCCGGAATTGTGAATAACGGACATACAATCCAAGTTAACGTGTT
AGAAGATTTTAACTGAACATCAAAGGCAAAGAATACCATCTTAAACAGTTTCATT
TCATGCTCCGTCTGAACATACAGTGAACGGCAAATATTATCCGCTTGAAATGCATCT
GGTCCATAAAGATAAAGATGGCAACATTGCAGTCATCGGAGTTTTCTTTAAAGAAG
GCAAAGCGAACCCGGAAGTTGATAAAGTTTTTAAAAACGCTCTGAAAGAAGAAGGA
AGCAAAGTGTTTGATGGCTCTATTAACATCAATGCGCTGCTTCCGCCGGTTAAAAAC
TACTACATACTGCGGAAGCTTAACAACACCGCCGTGTACAGAAGGCGTGCTGTG
GATTGTCCTTAAACAACCGATCACAGCTTCTAAACAACAGATTGAACTGTTTAAATC
AATCATGAAACATAACAACAATAGACCGACACAGCCGATTAAGTGTGCTATATCCT
GGAAAGCAATTAA

[00242] The foregoing discussion and examples have been presented for purposes of illustration and description. The foregoing is not intended to limit the aspects, embodiments, or configurations to the form or forms disclosed herein. In the foregoing Detailed Description for

example, various features of the aspects, embodiments, or configurations are grouped together in one or more embodiments, configurations, or aspects for the purpose of streamlining the disclosure. The features of the aspects, embodiments, or configurations, may be combined in alternate aspects, embodiments, or configurations other than those discussed above. This method of disclosure is not to be interpreted as reflecting an intention that the aspects, embodiments, or configurations require more features than are expressly recited in each claim. Rather, as the following claims reflect, inventive aspects lie in less than all features of a single foregoing disclosed embodiment, configuration, or aspect. While certain aspects of conventional technology have been discussed to facilitate disclosure of some embodiments of the present invention, the Applicants in no way disclaim these technical aspects, and it is contemplated that the claimed invention may encompass one or more of the conventional technical aspects discussed herein. The following claims are hereby incorporated into this Detailed Description, with each claim standing on its own as a separate aspect, embodiment, or configuration.

CLAIMS

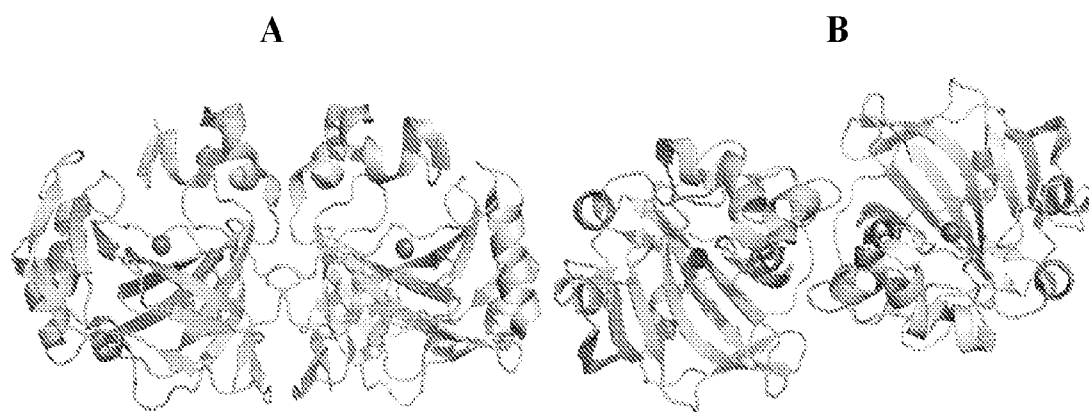
What is claimed is:

1. A non-naturally occurring carbonic anhydrase comprising at least one mutation that results in the substitution of at least one cysteine for at least one amino acid in a naturally occurring carbonic anhydrase; and wherein the non-naturally occurring carbonic anhydrase has increased activity at a temperature of greater than about 60 degrees Celsius when compared to the naturally occurring carbonic anhydrase.
2. The non-naturally occurring carbonic anhydrase of claim 1 wherein the increased activity is for more than about 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 24 hours, 44 hours, 48 hours, and 92 hours.
3. The non-naturally occurring carbonic anhydrase of claim 1 wherein the increased activity is at a temperature greater than 65, 70, 75, 80, 85 or 90 degrees Celsius.
4. The non-naturally occurring carbonic anhydrase of claim 1 wherein a nucleotide sequence encoding the non-naturally occurring carbonic anhydrase comprises a sequence that is greater than 70% identical to a sequence selected from the group consisting of SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 22, SEQ ID NO: 24 and SEQ ID NO: 26.
5. The non-naturally occurring carbonic anhydrase of claim 1 comprising an amino acid sequence that is greater than 70% identical to a sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 21, SEQ ID NO: 23 and SEQ ID NO: 25.
6. A method for CO₂ separation and CO₂ capture comprising the step of reacting CO₂ with a non-naturally occurring carbonic anhydrase comprising at least one mutation that results in the substitution of at least one cysteine for at least one amino acid in a naturally occurring carbonic anhydrase; and wherein the non-naturally occurring carbonic

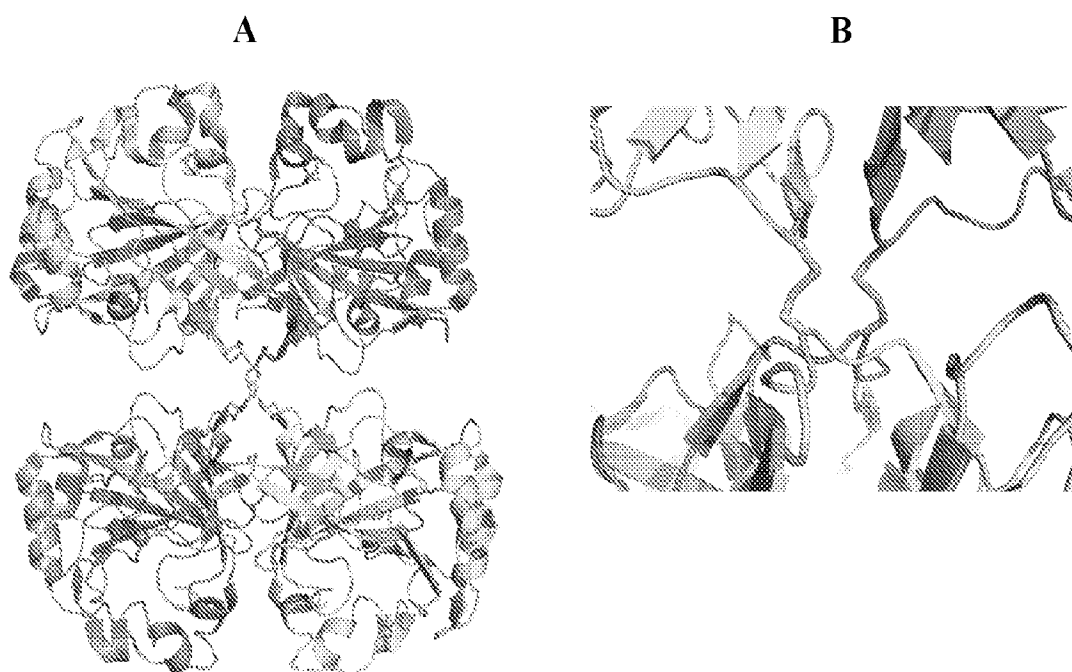
- anhydrase has increased activity at a temperature of greater than about 60 degrees Celsius when compared to the naturally occurring carbonic anhydrase.
7. The method of claim 6 wherein the step of reacting CO₂ with non-naturally occurring carbonic anhydrase is for more than about 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 24 hours, 44 hours, 48 hours, and 92 hours.
 8. The method of claim 6 wherein the step of reacting CO₂ with the non-naturally occurring carbonic anhydrase is at a temperature greater than 65, 70, 75, 80, 85 or 90 degrees Celsius.
 9. The method of claim 6 wherein the non-naturally occurring carbonic anhydrase comprises a nucleotide sequence that is greater than 70% identical to a sequence selected from the group consisting of SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 22, SEQ ID NO: 24 and SEQ ID NO: 26.
 10. The method of claim 6 wherein the non-naturally occurring carbonic anhydrase comprises an amino acid sequence that is greater than 70% identical to a sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 21, SEQ ID NO: 23 and SEQ ID NO: 25.
 11. A system for CO₂ separation and CO₂ capture comprising s non-naturally occurring carbonic anhydrase comprising at least one mutation that results in the substitution of at least one cysteine for at least one amino acid in a naturally occurring carbonic anhydrase; and wherein the non-naturally occurring carbonic anhydrase has increased activity at a temperature of greater than about 60 degrees Celsius when compared to the naturally occurring carbonic anhydrase; and wherein the system further comprises a support wherein the with the non-naturally occurring carbonic anhydrase is immobilized to the

support; and wherein the non-naturally occurring carbonic anhydrase is contacted with CO₂.

12. The system of claim 11 wherein the non-naturally occurring carbonic anhydrase has increased activity for more than about 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 24 hours, 44 hours, 48 hours, and 92 hours.
13. The system of claim 11 wherein the non-naturally occurring carbonic anhydrase reacts with CO₂ at a temperature greater than 65, 70, 75, 80, 85 or 90 degrees Celsius.
14. The system of claim 11 wherein the CO₂ to be separated and captured results from the combustion of fossil fuels or biomass.
15. The system of claim 11 further comprising a carbon capture unit wherein the carbon capture unit comprises an immobilized biocatalyst comprising an amino acid sequence that is greater than 70% identical to a sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 21, SEQ ID NO: 23 and SEQ ID NO: 25.



FIGs. 1A, 1B



FIGs. 2A, 2B

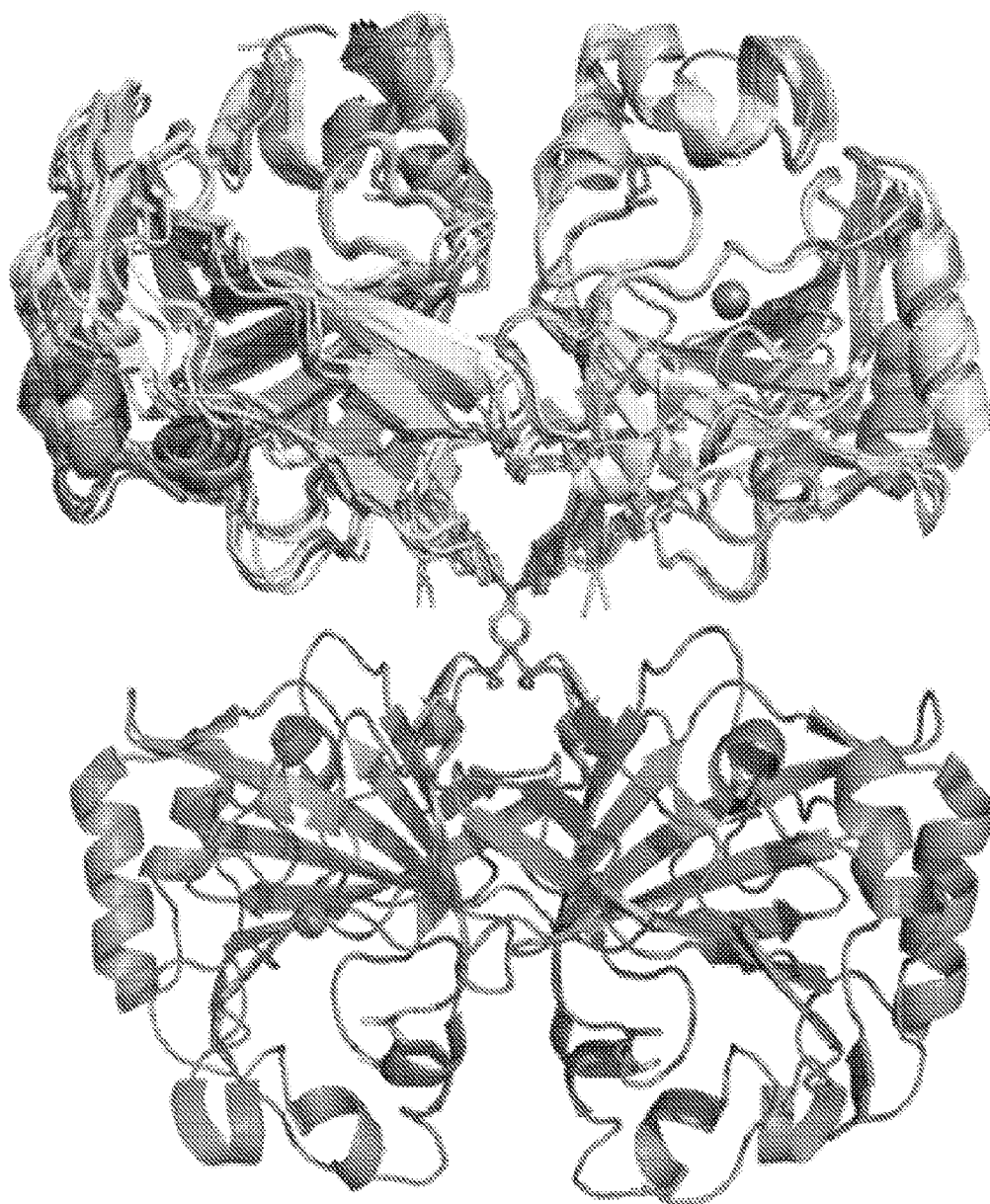


FIG. 3

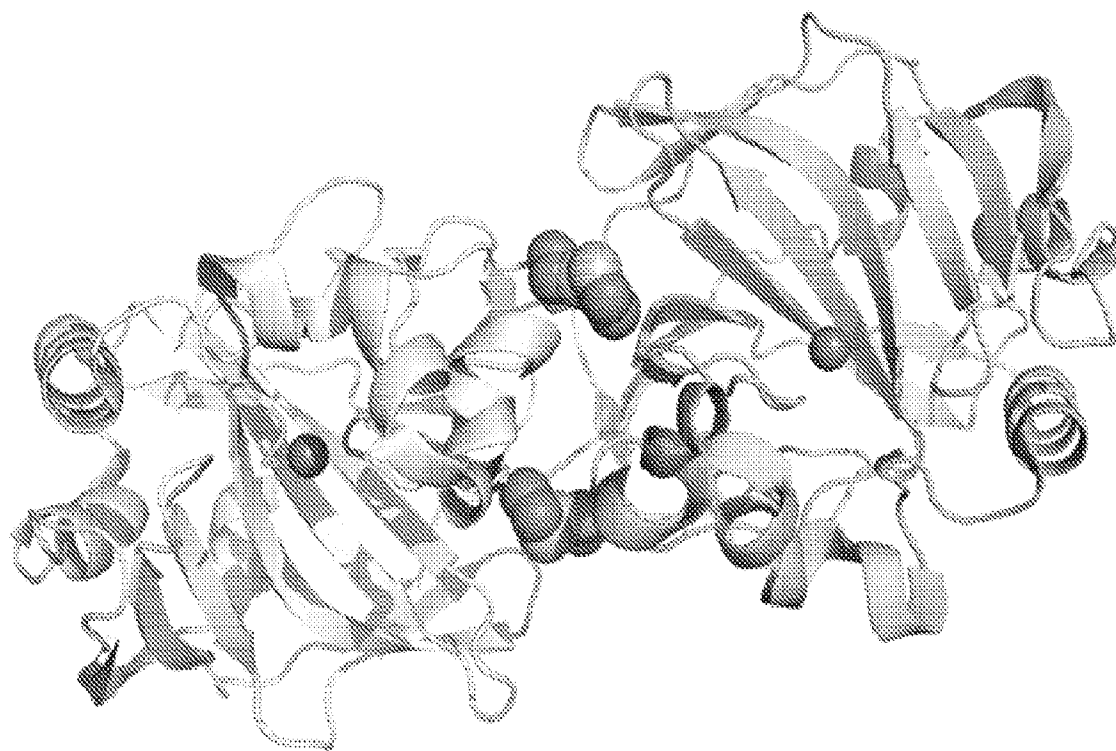


FIG. 4

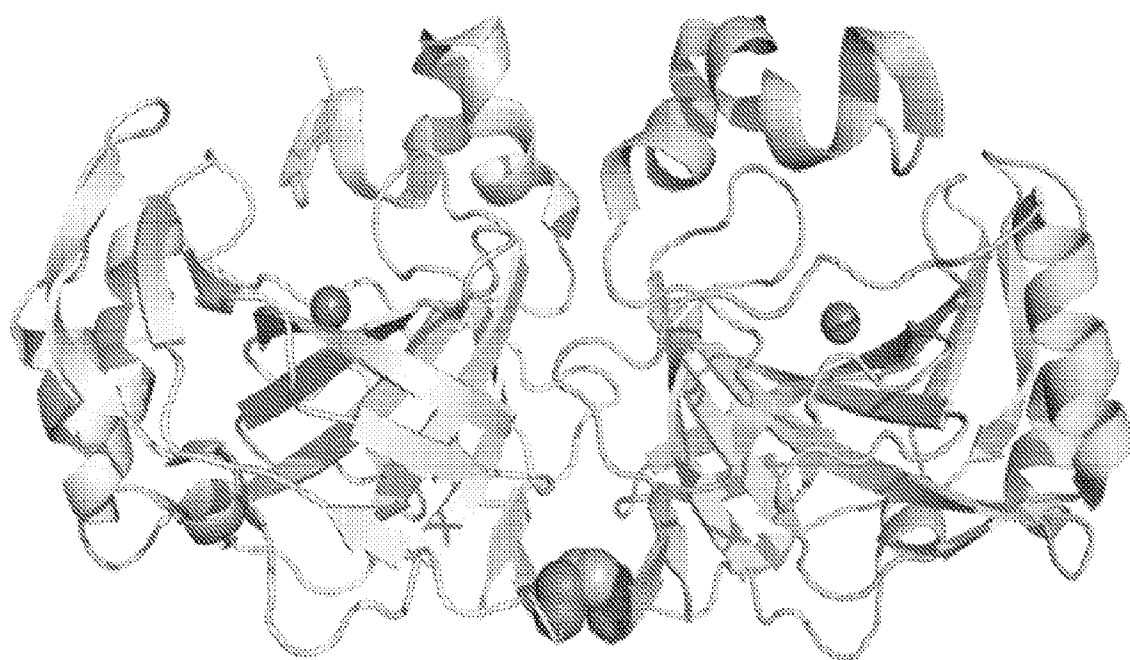


FIG. 5

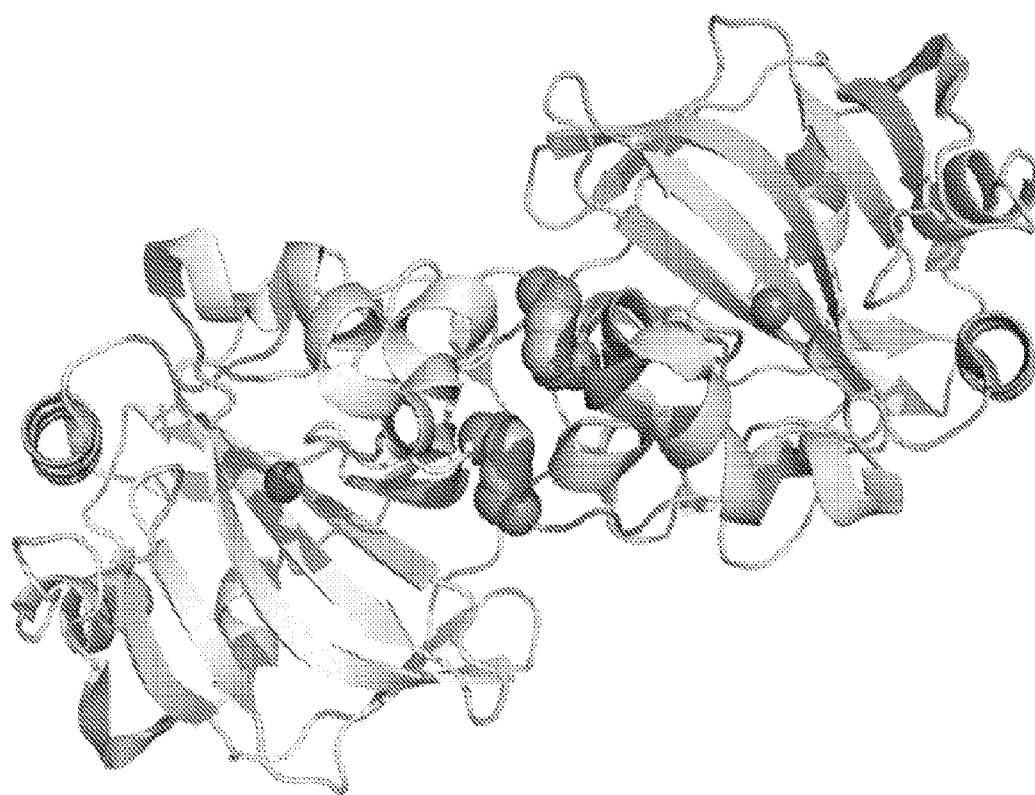


FIG. 6

FIG. 7

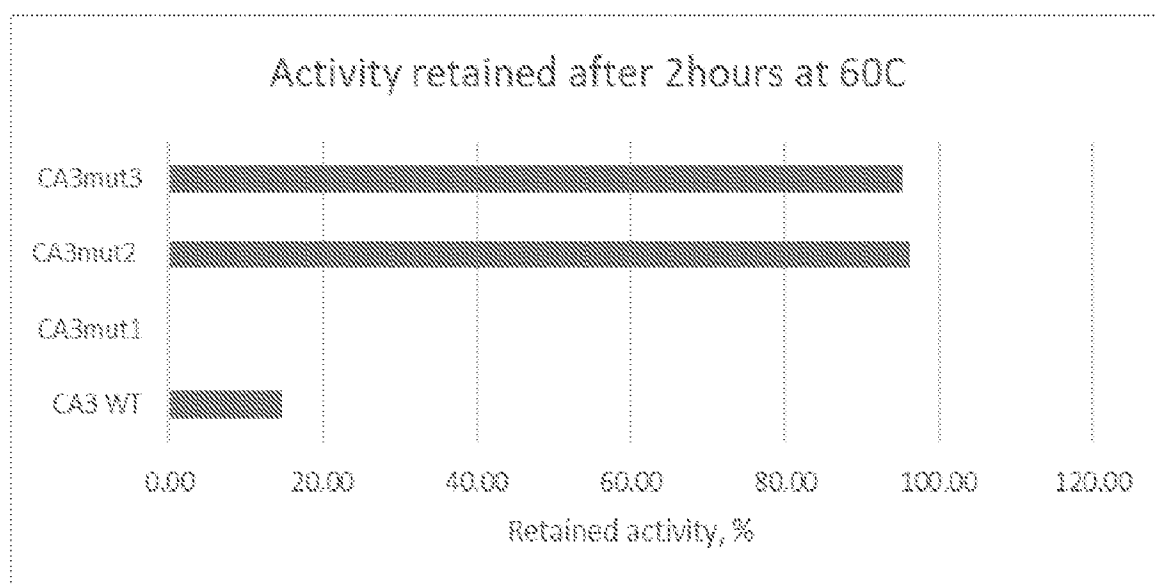


FIG. 8

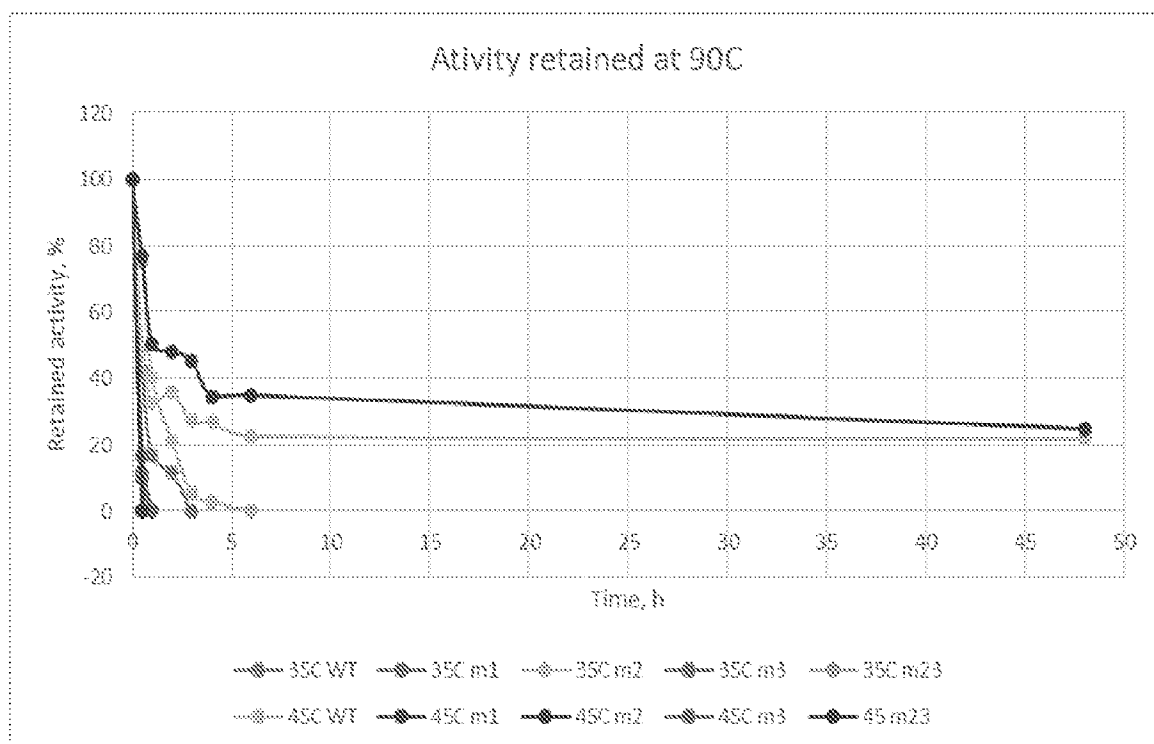


FIG. 9

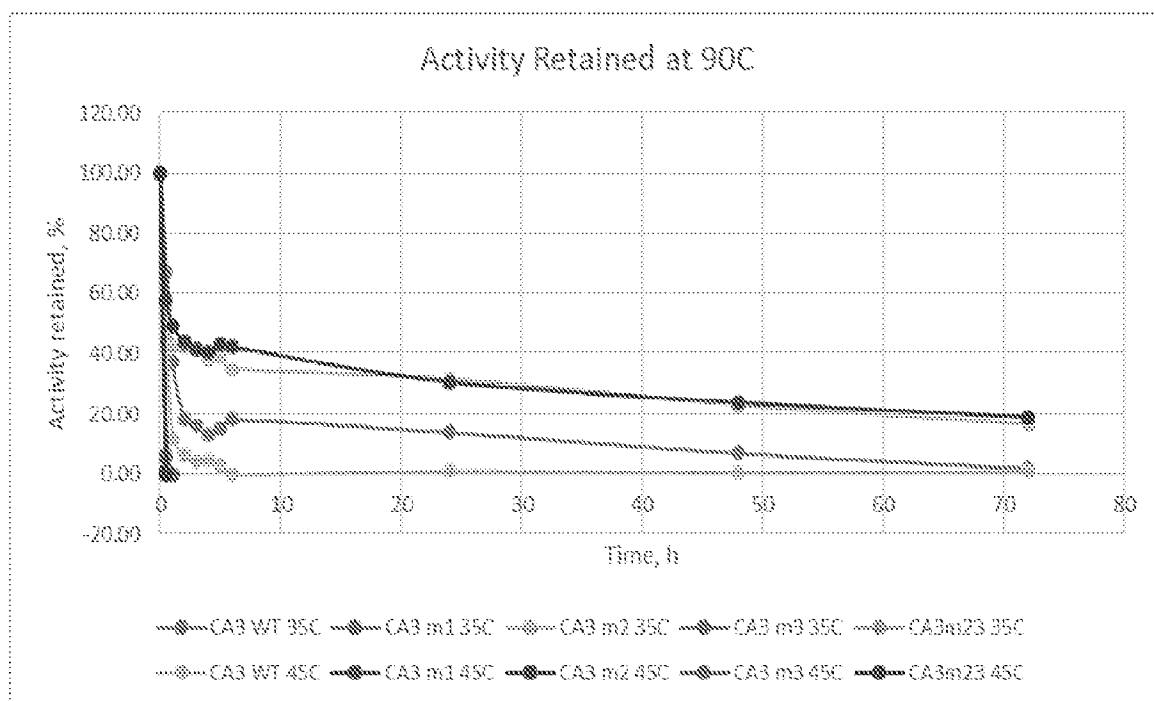


FIG. 10

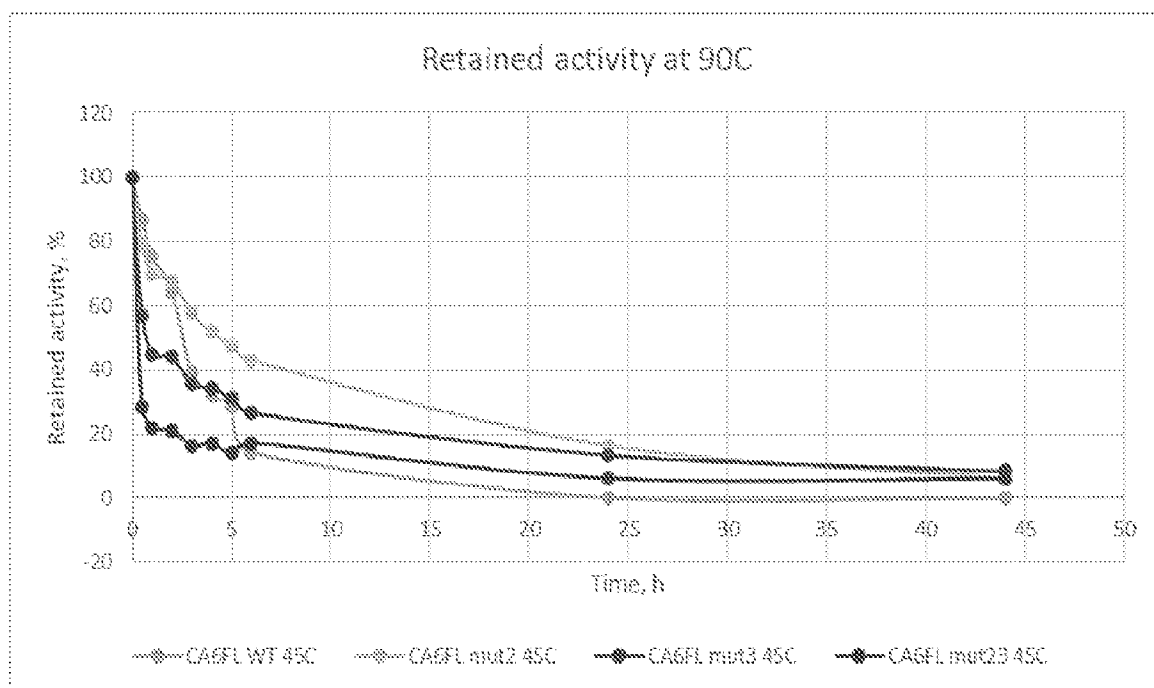


FIG. 11

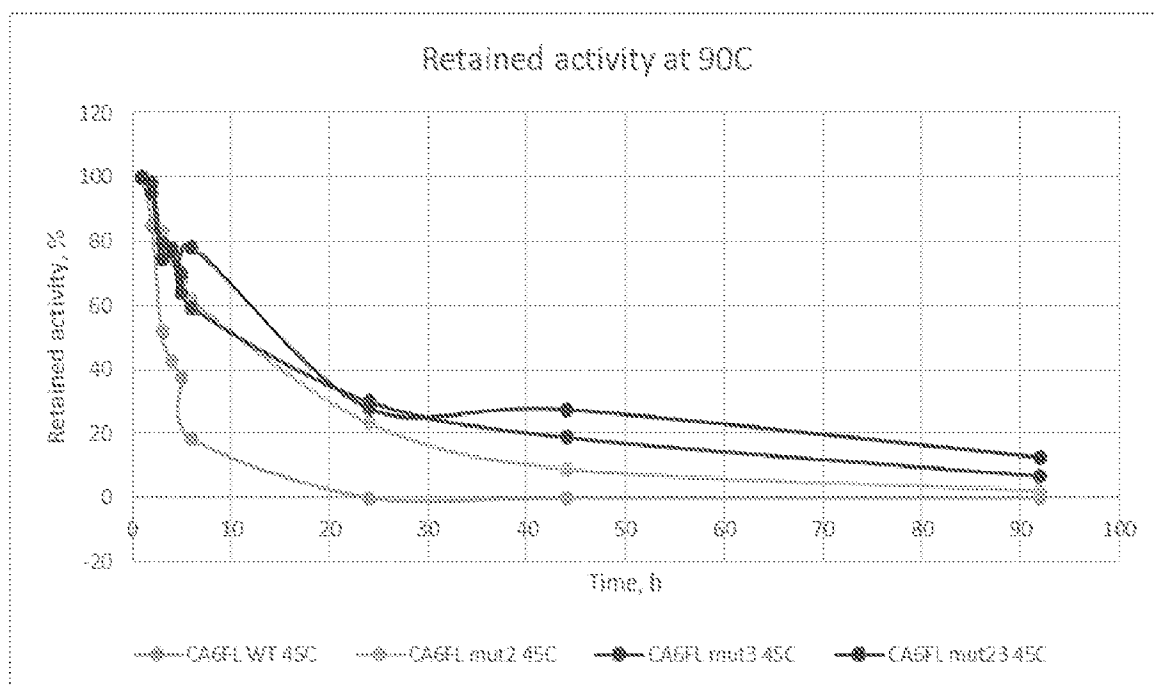


FIG. 12

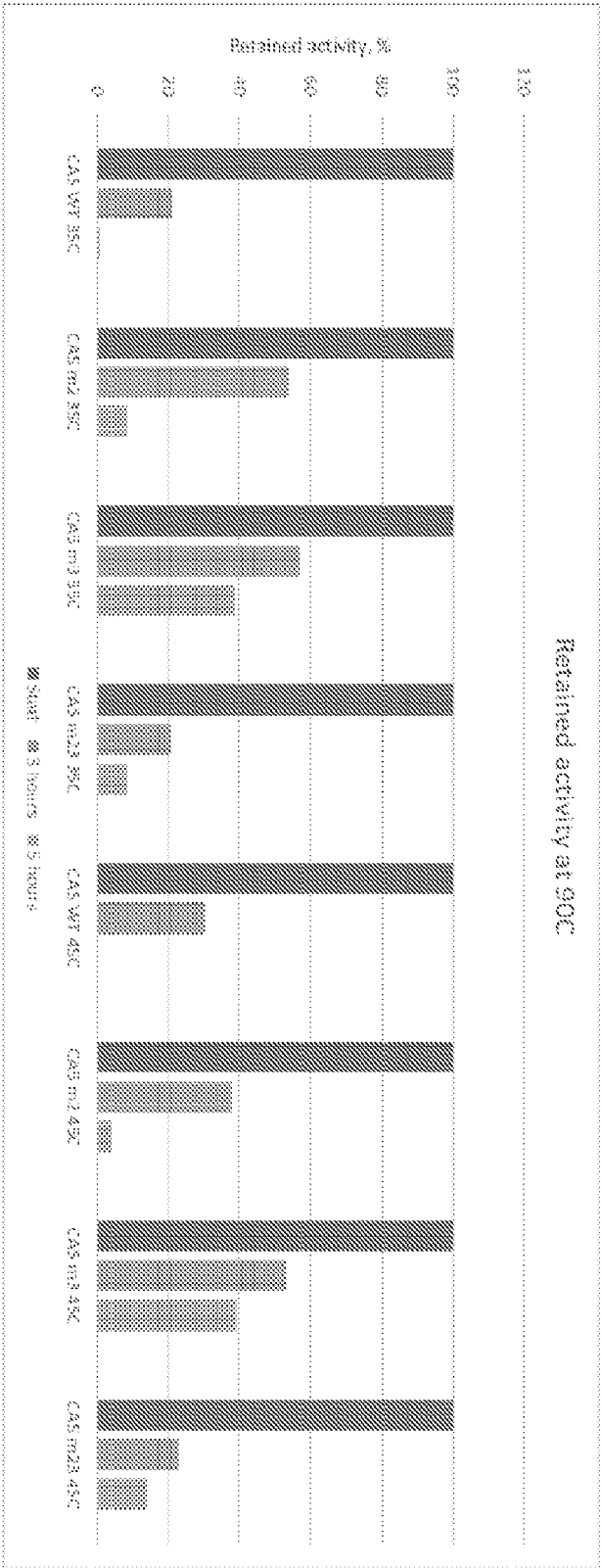


FIG. 13

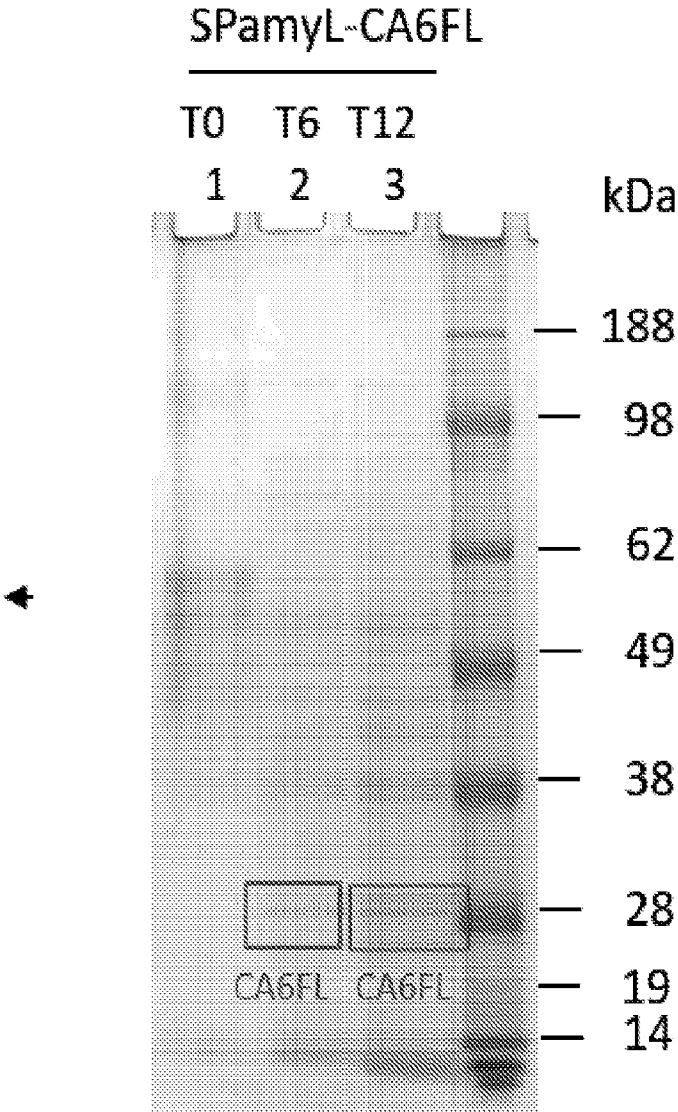


FIG. 14

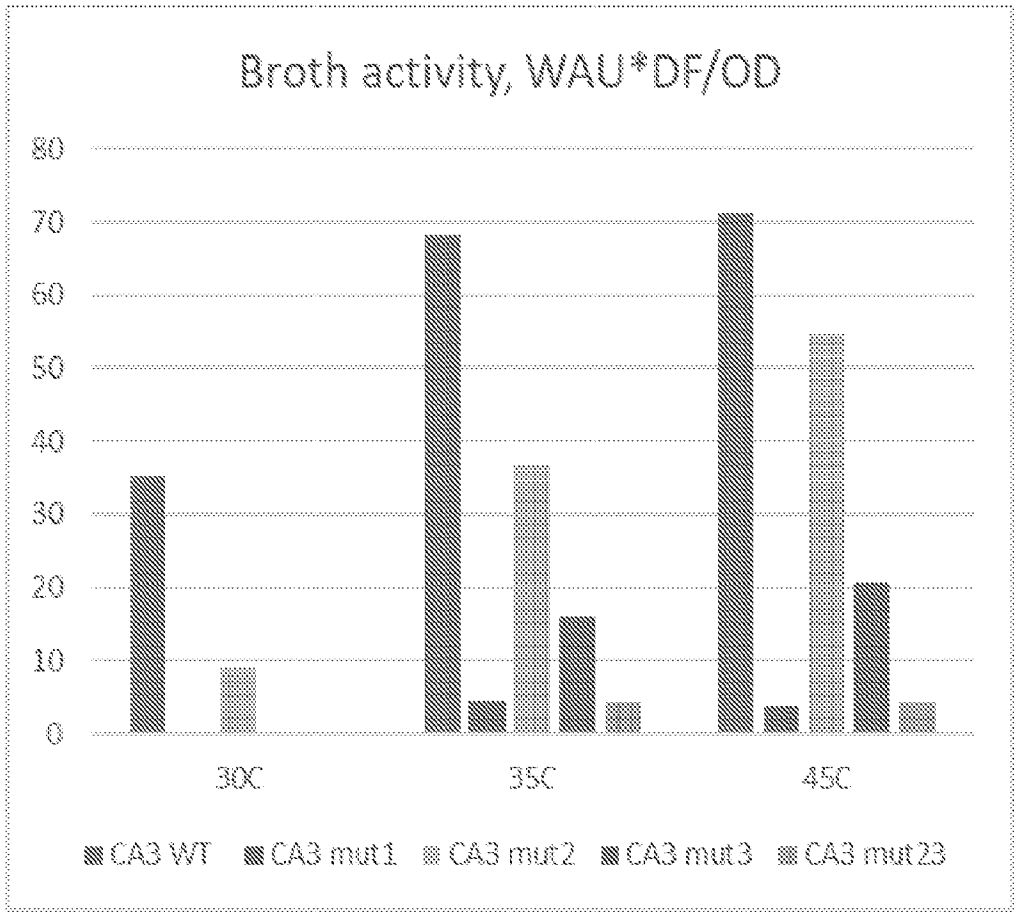


FIG. 15

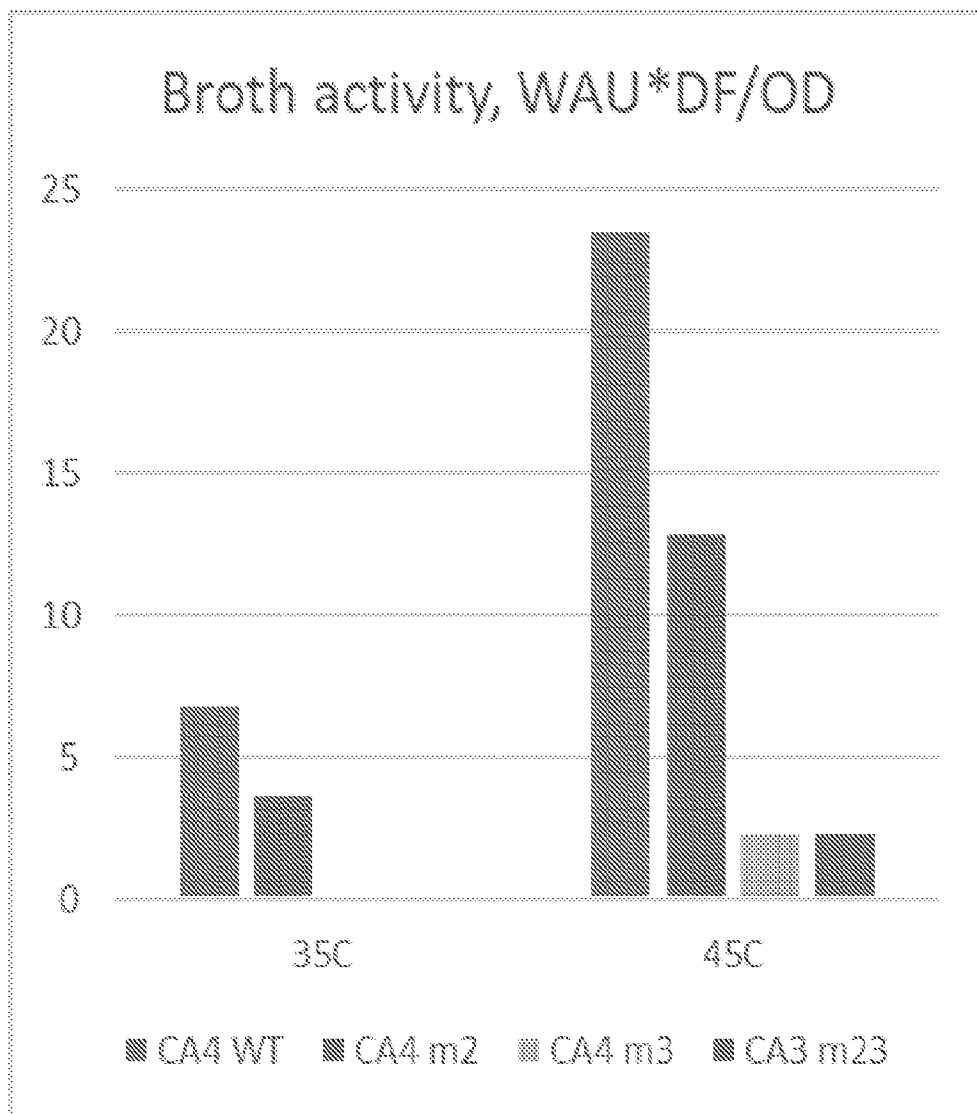


FIG. 16

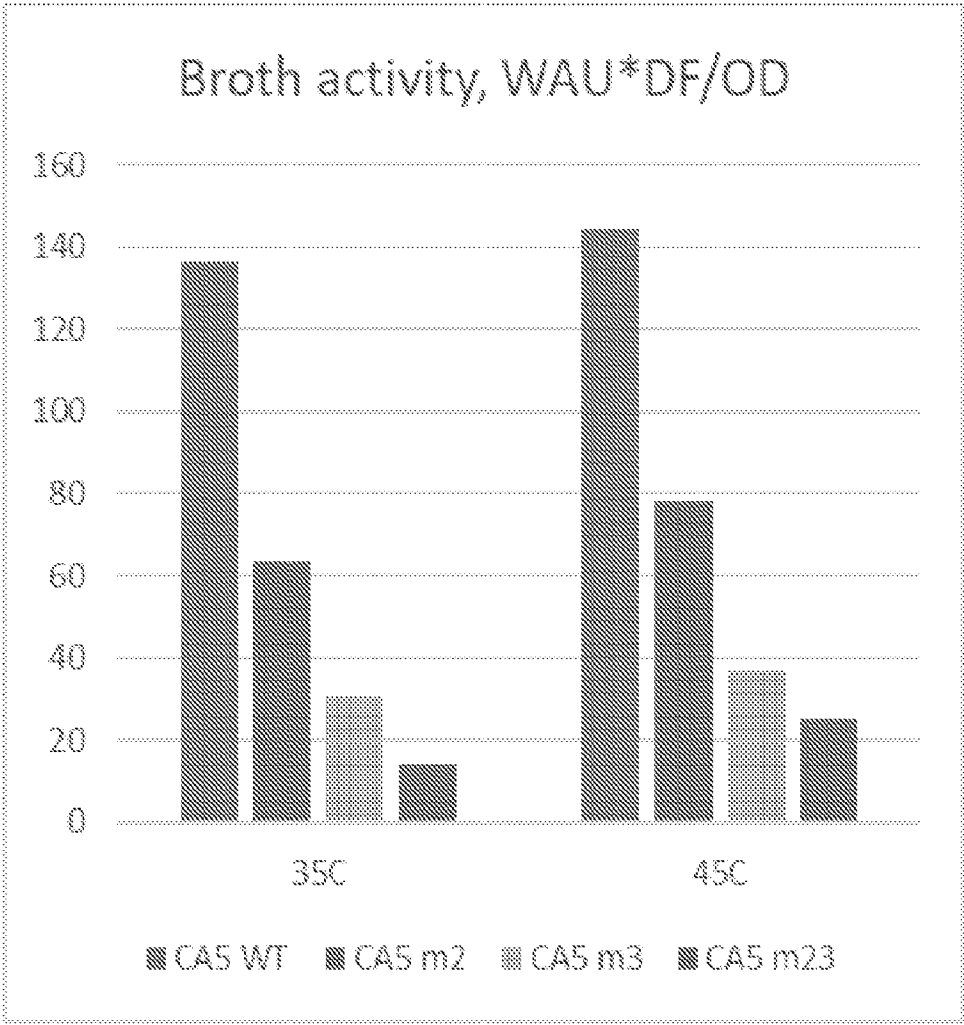


FIG. 17

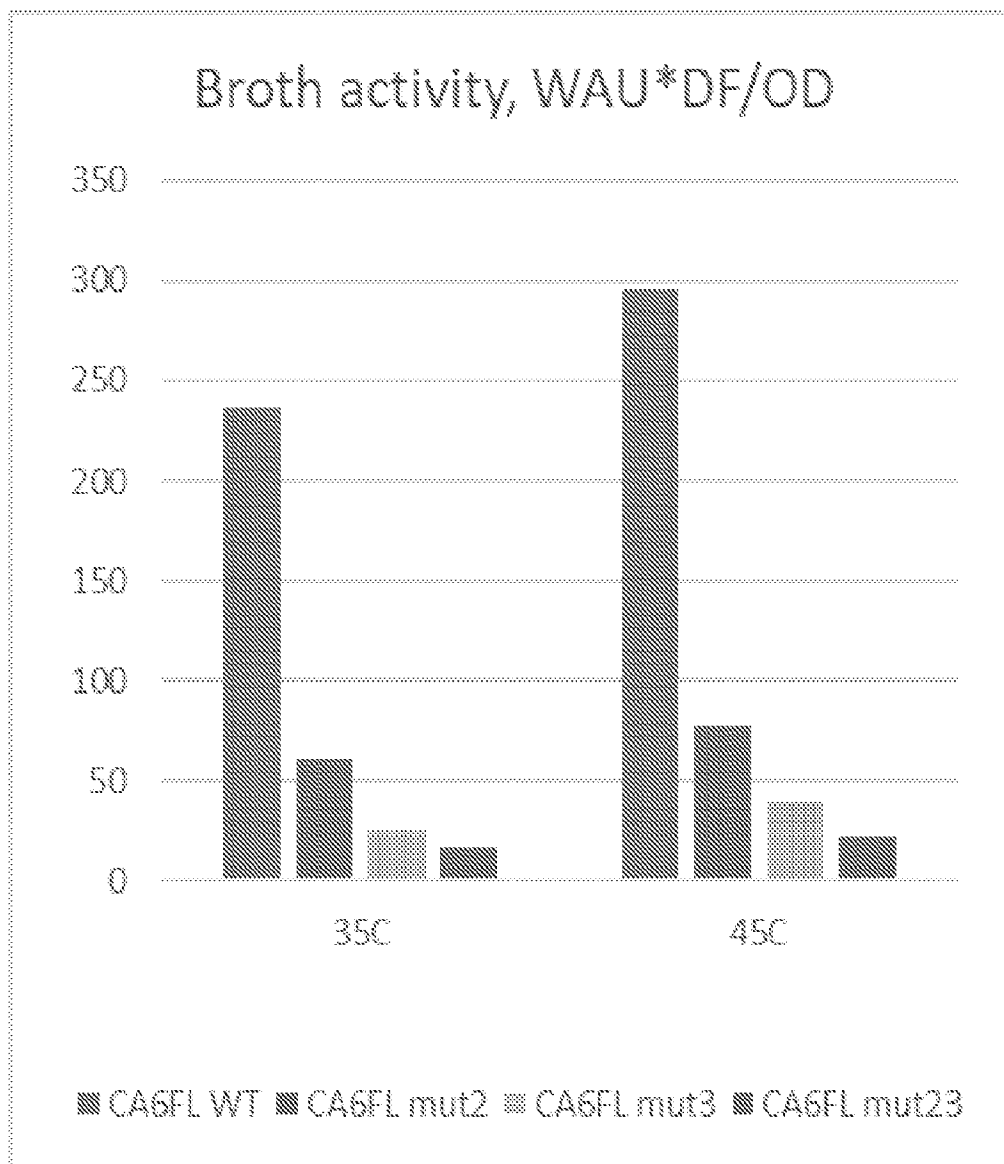


FIG. 18

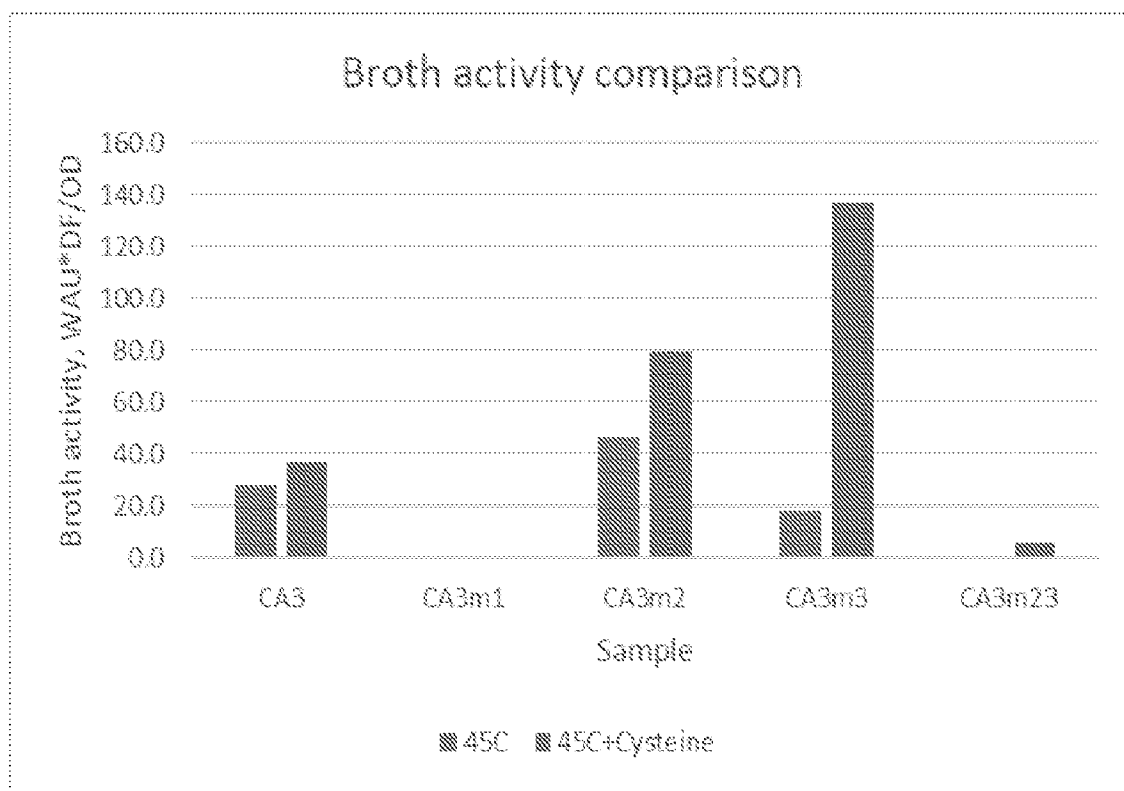


FIG. 19

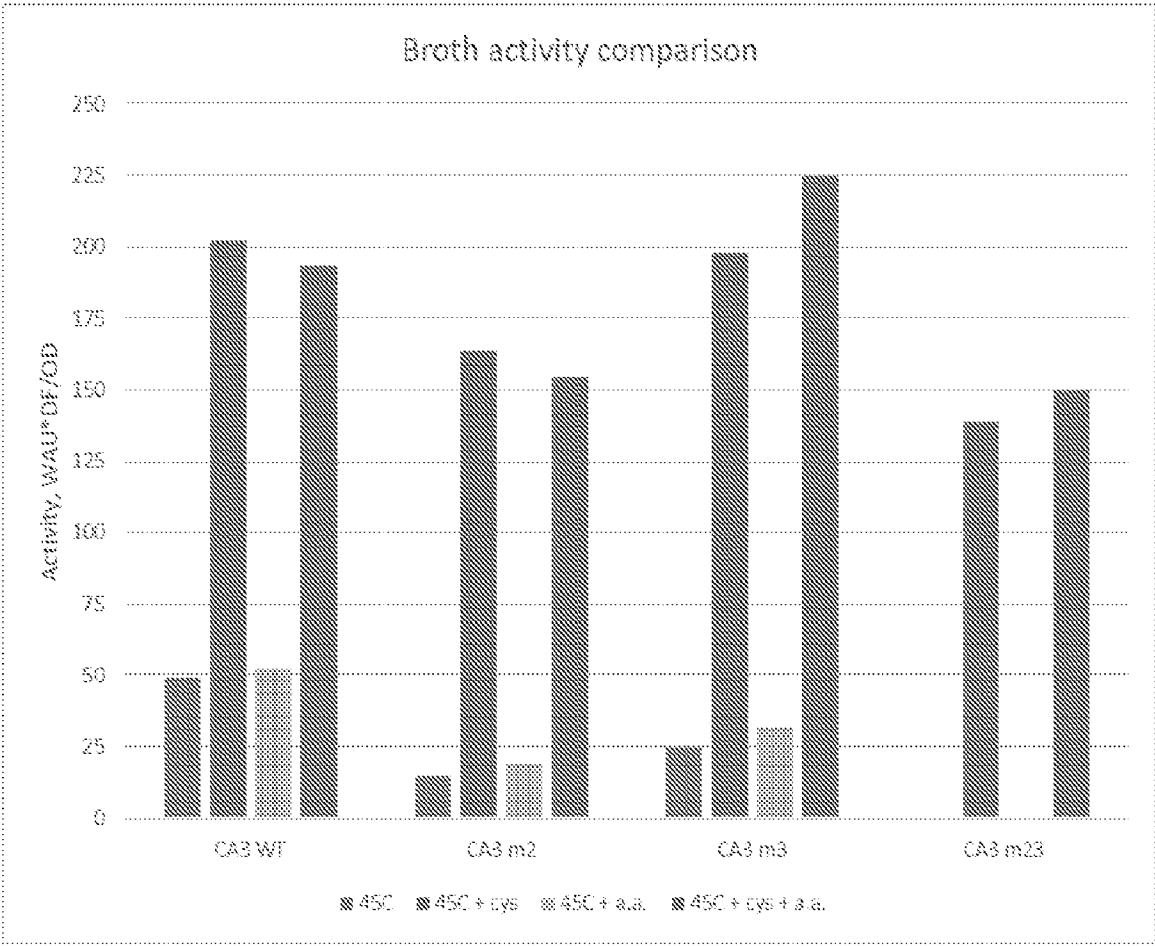


FIG. 20

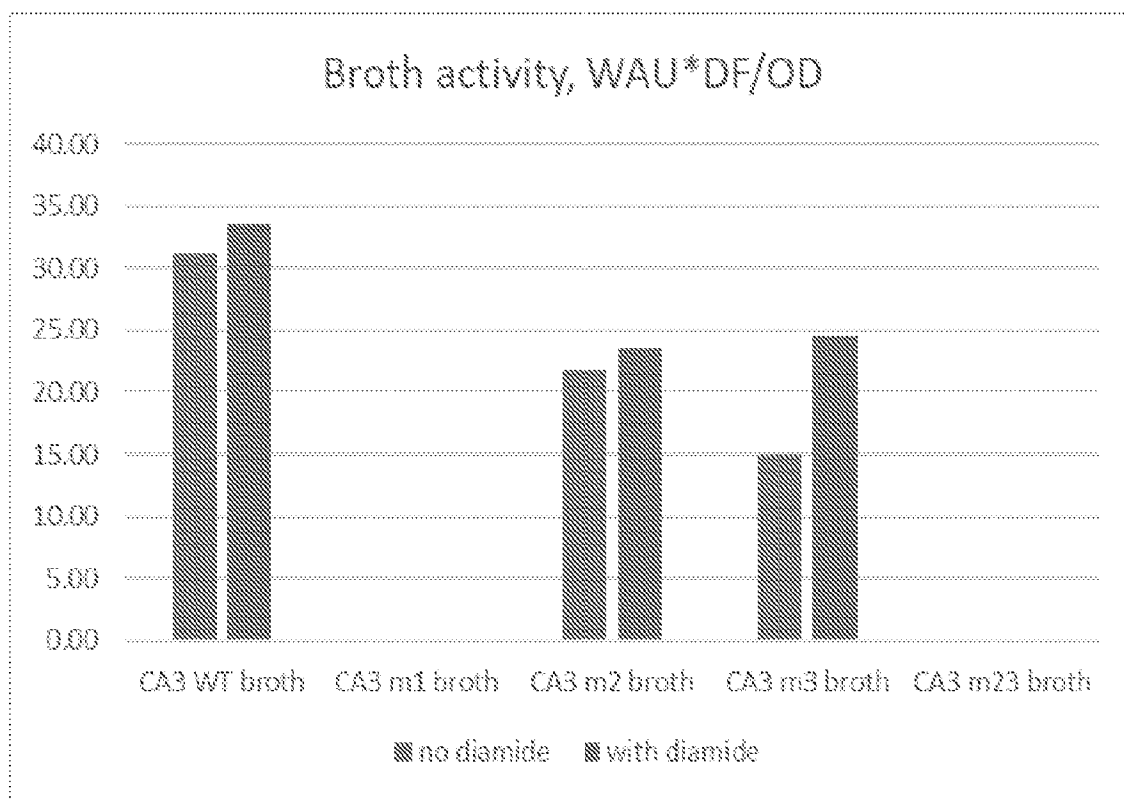


FIG. 21