Directed evolution of carbonic anhydrase to improve microbial induced calcium carbonate precipitation (MICP) efficiency

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Abstract

Microbial induced calcium carbonate precipitation (MICP) is an environmentally friendly way to produce inorganic material, and it`s considered as a feasible approach to resolve pollution problem and incompatible surface of inorganic and organic materials in repairation of concrete and soil. Urease and carbonic anhydrase are two key enzymes in MICP, which cooperate in the process. We express carbonic anhydrase in *Escherichia coli* and modify it by error-prone PCR, which is a widely used directed evolution technique. We obtain several mutants and measure their enzyme activity successfully. The structure of these mutants was predicted and analysed by means of bioinformatics.

Key words: directed evolution, MICP, carbonic anhydrase, error-prone PCR

Introduction

Error-prone PCR technology has a wide range of applications in vitro random mutation . It is currently the most extensively used in vitro directed evolution of proteins and plays an important role in improving the catalytic properties of enzyme molecules[1-3]. Error-prone PCR has been applied to improve the activity of glucanase, glycosyl trehalose synthase, starch hydrolase, lipase, laccase *β*, cellulase etal. What’s more, it is also very vital in guiding the study of the key amino acid residues that affect the catalytic properties of enzymes[4-9].

Microbial induced calcium carbonate precipitation (MICP) is a natural phenomenon that microorganisms form calcium carbonate deposits in their surrounding microenvironment through metabolism. As early as 1973, Boquet et al.[10] first discovered the phenomenon of soil microbial induced calcium carbonate precipitation (MICP). Most microorganisms in nature can generate cementation substances through their own metabolic activities, causing cementation between particles[11], thus improving the strength of soil and reducing its water permeability. Microbial cement has the characteristics of environmental protection, economy and efficiency, and has become a research hotspot in the fields of biology, civil engineering and environment. There are two key enzymes for biomineralization, namely urease and carbonic anhydrase[12]. Urease hydrolyzes urea to increase cell pH and carbonate concentration, forming alkaline conditions for calcium carbonate deposition. When Ca^{2+} adsorbed on the cell surface meets carbonate ions, it forms calcium carbonate precipitation and eventually biomineralization. Carbonic anhydrases (CA) are zinc containing metalloenzymes that can efficiently catalyze the reversible reactions between carbon dioxide, water, carbonic acid and H^+. Carbonic anhydrase can effectively promote biomineralization. In natural conditions, the hydration rate of CO_2 is quite low, which greatly limits the generation of calcium carbonate precipitation. However, the CO_2 hydration rate increases rapidly when the reaction above is catalyzed by carbonic anhydrase, reaching 10^8 times that in natural conditions.

Therefore, the rapid deposition of biologically induced calcium carbonate mineralization requires urease to decompose urea to increase the pH value of the environment, and carbonic anhydrase to promote the rapid hydration of CO_2 to form a large amount of CO_3^{2-}, thus forming calcium carbonate deposition with Ca^{2+} in the environment[12]. The mineralization and deposition cost of carbonic anhydrase mineralizing bacteria is low, there is no secondary pollution to the environment, and it can effectively store carbon dioxide, which has great potential in the research of environmental engineering fields such as mitigating global warming and other engineering fields[12]. At present, *Sporosarcina pasteurii* is the strain with the best biomineralization efficiency among known natural bacteria.

MICP research has been focused on screening strains with higher urease activity[13], in order to quickly produce CO_3^{2-} by accelerating the decomposition of urea, but the activity of carbonic anhydrase of the strain has been ignored. This study attempts to improve the activity of carbonic anhydrase by means of error-prone PCR to explore the effect of the increase of carbonic anhydrase activity on the mineralization rate of microbial cement.

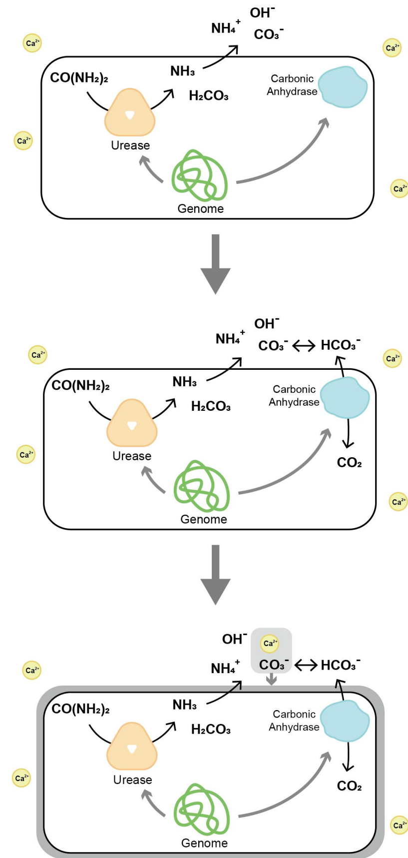

Fig.1a **A brief description of MICP induced by urease and carbonic anhydrase**

(1) Ca^{2+} ions in the environment are attracted to the bacterial cell wall due to the negative charged bacterial cell wall. Urease hydrolyses urea into ammonia and carbonate inside the cell. And then the ammonia and carbonate continue to hydrolyse spontaneously, forming ammonium, carbonate and hydroxide ions and releasing them into the environment, causing the pH in the environment to rise. (2) At high pH levels, carbonic anhydrase promotes the conversion of CO_2 to bicarbonate and further to carbonate. (3) The carbonate produced combines with Ca^{2+} in the environment to produce $CaCO_3$ precipitate on the bacterial cell wall.

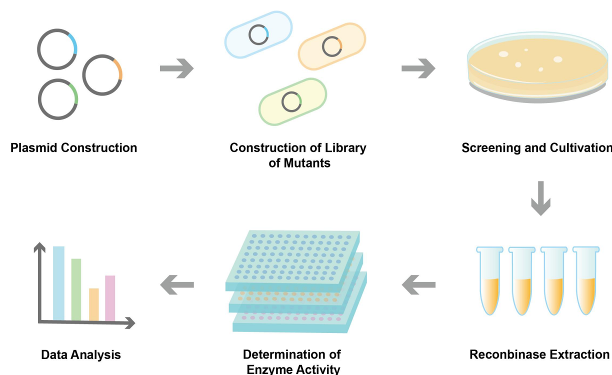

Fig.1b **How to create more efficient enzymes**

As shown in the left figure, in order to obtain a more efficient enzyme, after error-prone PCR, it is first necessary to construct a plasmid and transfer it into cells to construct mutant libraries, then culture and screen, extract the colony containing the recombinant plasmid, determine the enzyme activity, and then do some data analysis .

Result

1. Bioinformatics analysis of mASCA

1.1 Secondary structure and analysis of mASCA

The gene sequence analysis of mASCA was conducted with Phyer2 protein structure server, and the optimized carbonic anhydrase gene encodes 216 amino acids. The protein contains a total of 4 alpha helices and 14 beta folded fragments. The prediction results were with high confidence (Fig.2.1.1).

Fig.2.1.1 **Secondary structure of mASCA**

1.2 Homology modeling and 3D structure prediction of mASCA

The results of homology modeling of mASCA using Phyer2 protein structure server showed 50% sequence identity between the protein and PDB database template c6ekiA and with 100 % confidence level (Fig.2.1.2). Since the homology modeling method is based on sequence homology comparison, it is more effective for the simulation of sequences with sequence similarity greater than 30%, and therefore our result has a high confidence level.

1.3 Evolutionary tree and evolutionary relationship analysis of mASCA

The Bootstrap method was selected as the test method for tree construction. At the same time, parameter set as 500, which means the software constructed 500 corresponding trees (Fig.2.1.3). It means that several segments of sequences in the branch with corresponding times and frequencies are similar in evolutionary speed. On the Bootstrap consensus tree, the number at the node indicates how many percent of the trees have branches of the tree through step size test, which reflects the credibility of the branch. Most of the phylogenetic trees constructed at present are at 70 or above, which means the branch reliability is high. The position of the target species protein in the phylogenetic tree is indicated by red dot. The amino acid sequence of *Gammaproteobacterial* bacterium carbonic anhydrase is speculated to be the most distant in evolutionary relationship with the target species, and the most distant in genetic relationship. These fifteen species form a rooted tree when phylogenetic tree is constructed, which can be used to infer the common ancestor and evolutionary direction, and has a hierarchical structure. If a rooted tree cannot be formed, the relationship between different species can also be found by consulting the literature, and outgroup species can be assigned to determine the root node. The carbonic anhydrase of the target species *Aliivibrio salmonicida* has the closest evolutionary relationship with the protein corresponding to *Aliivibrio fischeri*, and the furthest evolutionary relationship with *Gammaproteobacterial* bacterium. It is in a relatively late evolutionary position, and the gene separation is late from other proteins.

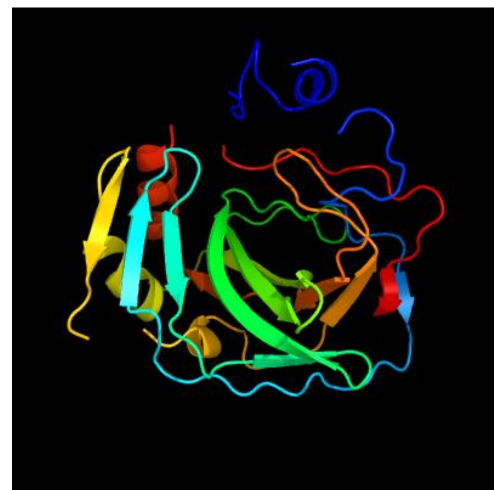

Fig.2.1.2 **3D structure of mASCA**

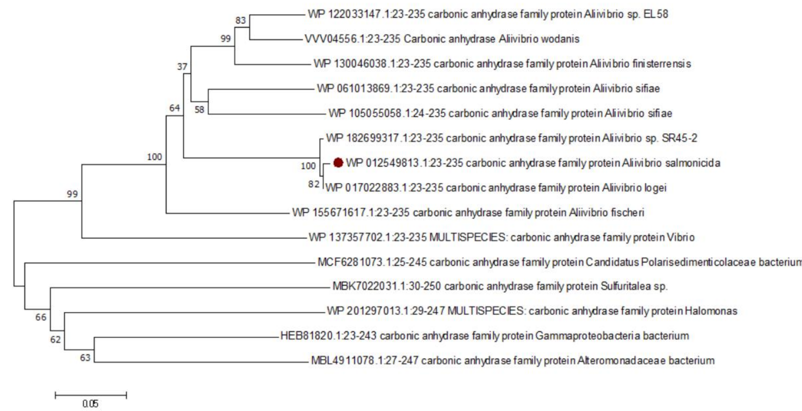

Fig.2.1.3 **Evolutionary relationships of carbonic anhydrase of *Aliivibrio salmonicida* and other relevant species.**

3.2 Construction and profiling of the plasmid

The full-length DNA sequence of the 705-bp gene encoding CA (Accession No:YP_002262544) from the *Gramnegative* marine bacterium, *A. salmonicida*, was chemically synthesized, in which the codon was optimized for expression in *E. coli*. The expression products have been validated[14]. The DNA sequence of the mature mASCA, was amplified by PCR using the mASCA-pF and mASCA-pR primers, and the resultant product was digested sequentially with EcoRI and HindIII and ligated into the corresponding sites of the pETDuet-1 plasmid to construct the mASCA expression vector, pETD-mASCA (Fig.2.1.4). The buffer and the incubation temperature are the same. After the cloning was verified by sequencing, pETD-mASCA was transformed into *E. coli* BL21.

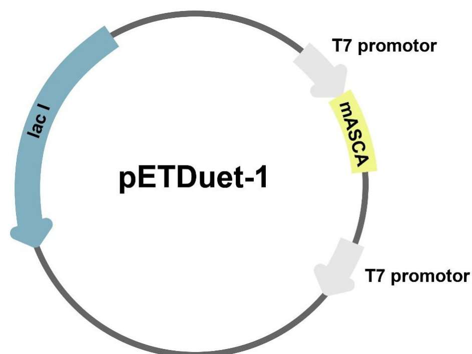

Fig.2.1.4 **pETD-mASCA**

2. Analysis of physical and chemical properties of mASCA

2.1 Analysis of the optimum Ca^{2+} concentration for mASCA expression

According to Fig.2.2.1a, the change of Ca^{2+} concentration has no linear influence on the growth of bacteria, and different concentrations have little influence on the growth of bacteria. Therefore, the influence of the change of Ca^{2+} concentration on the amount of bacteria can be ignored. However, different concentrations of calcium ions have certain effects on the specific activity of carbonic anhydrase. According to our Fig.2.2.1b, when the Ca^{2+} concentration was 110mg/L, the specific enzyme activity was the highest. In conclusion, 110mg/L Ca^{2+} concentration should be selected as the value of the subsequent evolution and expression process as the most suitable choice.

3. Analysis of error-prone PCR results

3.1 Condition optimization of error-prone PCR

Controlled error-prone PCR Kit (No. ZY-160903) was used to preform sequential error-prone PCR on mASCA. The initial set of number of cycles is 45 and the expected mutation value is 6 per 1000bp. The results of error-prone PCR products

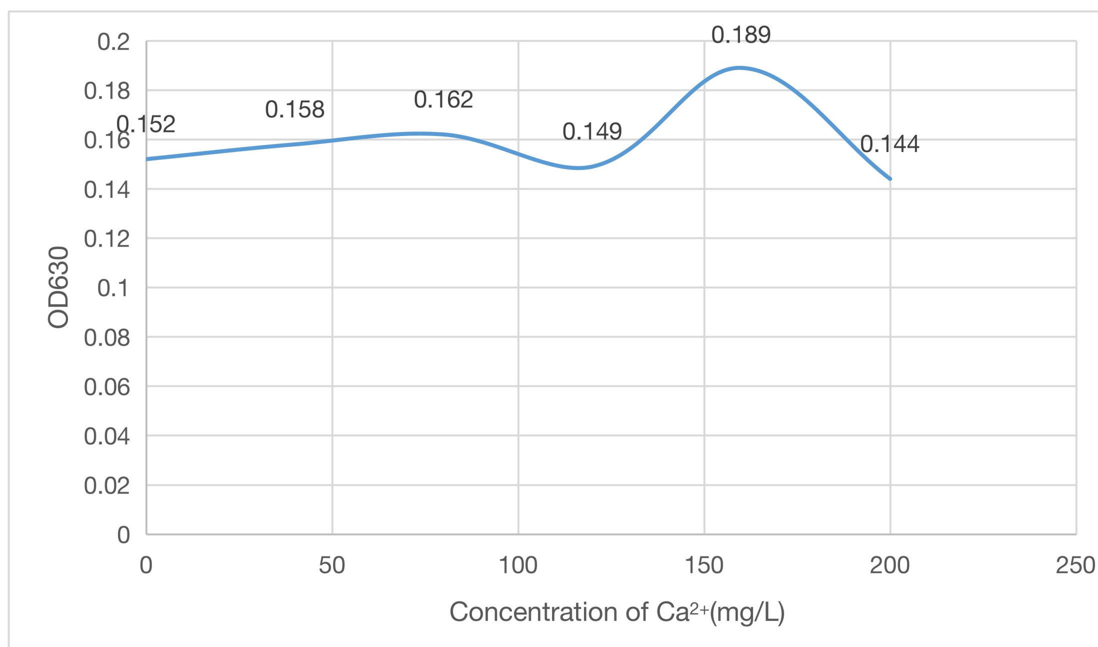

Fig.2.2.1a **Effect of Ca^{2+} concentration on bacterial growth**

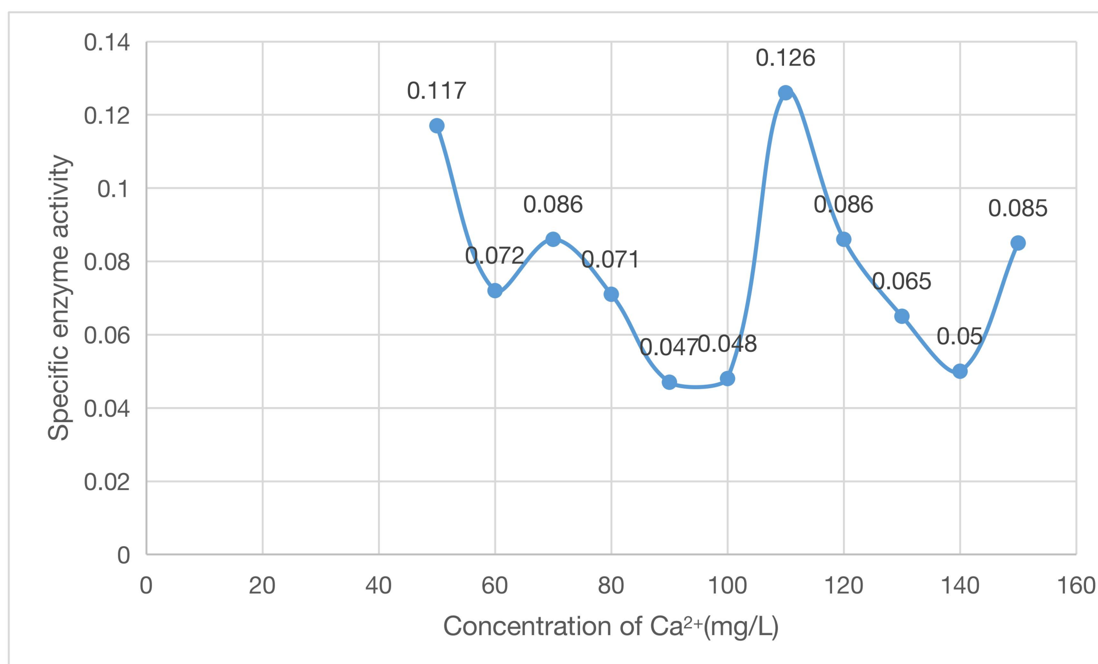

Fig.2.2.1b **Effect of Ca^{2+} concentration on specific enzyme activity of CA**

after agarose gel electrophoresis are shown in Fig.2.3.1a. The products was digested sequentially with EcoR Ⅰ and HindⅢ and ligated into the corresponding sites of the pETDuet-1 plasmid to construct the vector(Fig.2.3.1b) and the vector was transformed into *E. coli* BL21. It is possible that too many cycles resulted in an increase of non-specific products and therefore failed to be transformed into *E. coli*. After changing the number of cycles to 35, the error-prone PCR products were successfully transformed into *E. coli* BL21 (Fig.2.3.1c).

3.3 Enzymatic activity analysis of expressed proteins after error-prone PCR

We determined the activity of recombinant enzymes obtained from 10 mutant bacterial strains and the original bacterial strain (Fig.2.3.2a).

No.0 is the original bacterial strain while the No.1 to No.11 are the mutant bacterial strains. The y-axis of the chart is OD348(3min) -OD348(0min), which represents the activity of recombinase. The enzyme activity of original strain was 0.043, and 6 of the 10 strains we obtained increased enzyme activity. Among them, the activity of enzyme No.2 was significantly increased, changing from 0.043 to 0.281, and the activity of enzyme increased by about 6 times. In conclusion, we obtained an enzyme with significantly improved activity by error-prone PCR. We will sequence its corresponding gene fragment to find out the changes, and will analyze the changes in the secondary structure and tertiary structure of the protein to elaborate the reason for its enzyme activity improvement.

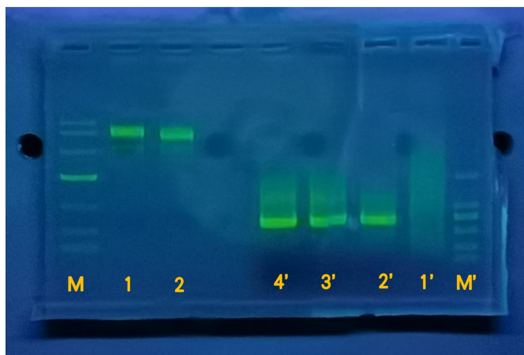

Fig.2.3.1a **Product electrophoresis results (45 circles)**

M: Marker10000

1: Plasmid pETDuet-1 electrophoresis product

2: Plasmid pETDuet-1 electrophoresis product

M': Marker2000

1': Error-prone PCR products with a concentration of 1ng/μL as template

2': Error-prone PCR products with a concentration of 1ng/μL as template

3': Error-prone PCR products with a concentration of 10ng/μL as template

4': Error-prone PCR products with a concentration of 10ng/μL

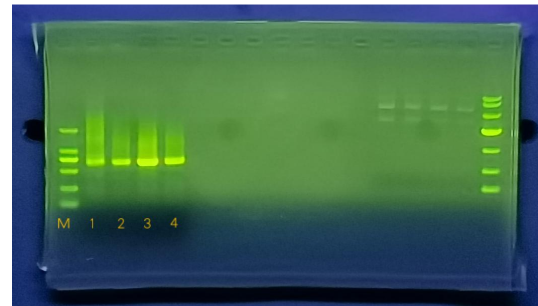

Fig.2.3.1b **Product electrophoresis results (35 circles)**

M: Marker2000

1: Error-prone PCR products with a concentration of 1ng/μL as template

2: Error-prone PCR products with a concentration of 1ng/μL as template

3: Error-prone PCR products with a concentration of 10ng/μL as template

4: Error-prone PCR products with a concentration of 10ng/μL

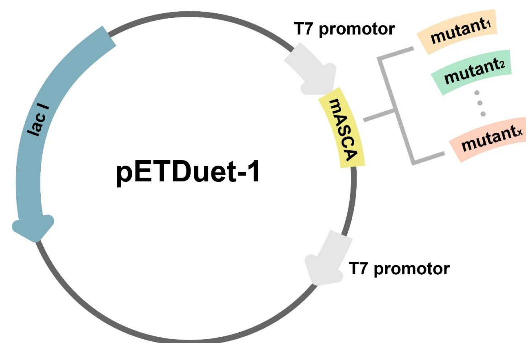

Fig.2.3.1c **Plasmids for mutant constructs**

Discussion

In this experiment, the carbonic anhydrase gene was successfully expressed and mutated. The activities of eleven strains and the activities of original carbonic anhydrase were measured after the error-prone PCR mutation. Although the activities of carbonic

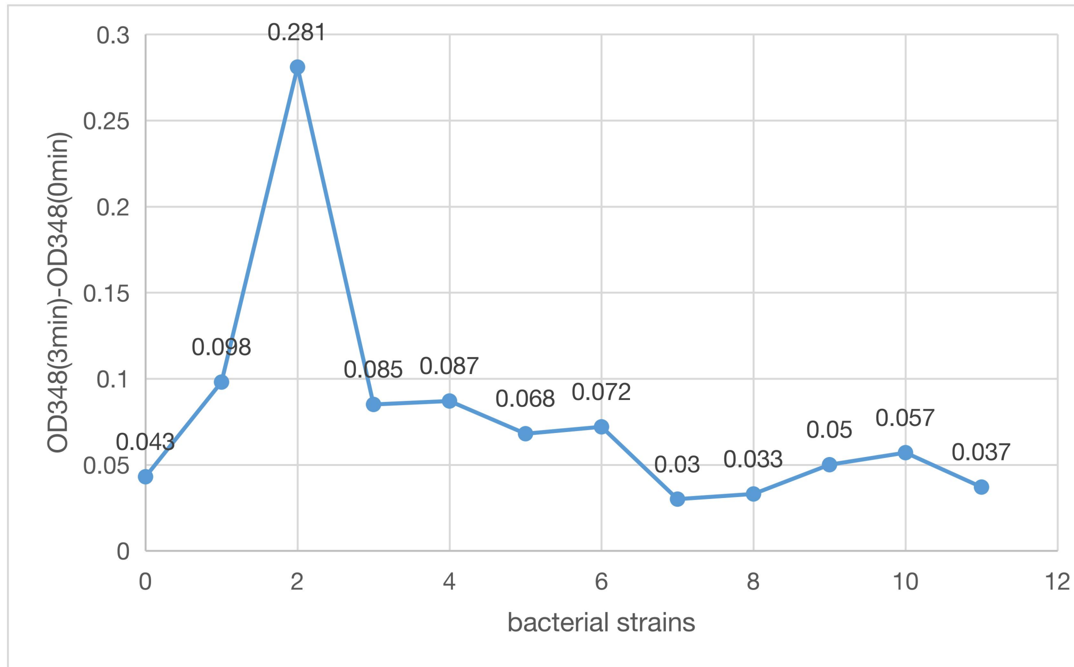

Fig.2.3.2a **Values of the enzyme expressed by the mutant bacterial strains and by the original bacterial strain.**

anhydrase of some strains after the mutation decreased compared with the original strains, the activities of Ep1, Ep2, Ep3, Ep4, Ep5, Ep6 and Ep10 increased to a certain extent compared with the original strains. Among them, the enzyme activity of Ep2 ncreased greatly, indicating that this method was effective in the evolution of carbonic anhydrase. Then, we also predicted the structure of the mutant and analyze the evolutionary relationship of mASCA.

At present, many researchers have used genetic engineering methods to heterologous express urease from different species. In this experiment, we also showed that the expression of carbonic anhydrase in Escherichia coli is feasible, and previous studies have shown that urease is also feasible. Not only that, we aslo mutated the carbonic anhydrase gene in *Escherichia coli* based on the error-prone PCR technology, and screened the mutant strains from the established carbonic anhydrase mutation library.

The detection results showed that the mutant carbonic anhydrase had higher enzyme activity, which proved the feasibility of mutating the carbonic anhydrase expressed in *Escherichia coli* using error-prone PCR technology. The earliest discussion on the synergistic effect of urease and carbonic anhydrase in MICP came from the article of Achal et al. 2011[15].

In their paper, they demonstrated that carbonic anhydrase also plays an important function in MICP. In their discussion, they mentioned that the maximum activity of both urease and carbonic anhydrase appeared after about 120 hours of culture, which might indicate a relationship between the two enzymes. And compared to *Sporosarcina pasteurii*, *Escherichia coli* can form biofilms to attach to living tissues or non-living surfaces, and the bacteria can be wrapped in biofilm to form a large number of bacteria aggregation. In previous studies on urinary calculi, researchers used *Escherichia coli* as engineering bacteria to observe the growth of calcium carbonate crystals in the system.

The metabolism of the modified *Escherichia coli* can produce urease to decompose urea, and the carbonate ions produced after urea decomposition combine with the free metal cations in nature to form gelation crystals. The above two points prove that the modified *Escherichia coli* is suitable for microbial induction of calcium carbonate precipitation.

So,in the future, we will sequence the mutants in order to identify the mutation sites and introduce mutated carbonic anhydrase gene and non-mutated carbonic anhydrase gene into *Escherichia coli*, and even use EvolvR system to conduct multi-window directed evolution of the urease gene, so that the evolved urease and carbonic anhydrase can cooperate in MICP. It may improve the relatively low urease activity in *E. coli* and improve the efficiency of bacterial mineralization, which is more efficient and suitable for laboratory research or ecological engineering, civil engineering and other fields.

Materials and Methods

1. Strains and plasmids

The amino acid sequence of CA was obtained from the genome sequence of Vibrio salmonicida, a strain of bacteria from the ocean. The nucleotide sequence coding the mature enzyme was optimized for expression in *E. coli* BL21(DE3) and expressed using the pETDuet-1 vector that contains the ColE1 as the origin of replication, an ampicillin resistance gene as the selection marker, the T7 promoter and the lac operon. The constructed plasmid was transformed into *E. coli* BL21(DE3) by receptive state transformation.

2. Gene cloning and expression vector construction

Analysis of the Multiple Cloning Site (MCS) of the pETDuet-1 fragment vector sequence showed that the best double enzyme digestion sites on MCS were HindⅢ and EcoRⅠ. SnapGene was used to design a pair of added protective bases (pF:GC, pR:CC) primers for amplifying mASCA genes,

pF:5'-GCGAATTCATGTCTGAATGGTCTTAC TCTGG - 3' (Tm:59℃)(BamHⅢ endonuclease site) and

PR: 5'-AAGCTTTCATTTCAGGATCGGCCC-3' (Tm:55-56℃)(EcoRⅠ endonuclease site).

Using the above primers, mASCA gene was amplified by PCR reaction system with 50μL solution using the synthesized mASCA gene as template. The reaction system is as follows:

Mix(2 ×)	25 μL
pR	1 μL
pF	1 μL
bacteria solution	3 μL

After predenaturation at 95℃ for 5min, 30 cycles of reaction were performed: denaturation at 95℃ for 30s, annealing at 58℃ for 30s, extension at 72℃ for 1min, and finally extension at 72℃ for 10min. The PCR products were examined by electrophoresis on 1% agarose gel.

The PCR product and plasmid pETDuet-1 were purified by DNA purification kit and double digested with BamH Ⅲ and EcoR Ⅰ. The reaction system was as follows:

Gene	20μL
10×NEB	5μL
BamHⅢ enzyme	1μL
EcoRI enzyme	1μL

The reaction was performed at 37℃ for 1h and then terminated at 80℃ for 20min. The digested products were recovered by DNA purification kit and quantified. After that, the two products were reacted with T4 DNA ligase at room temperature for 30min or overnight at 16℃, and then transformed into competent *E. coli* BL21(DE3) cells, incubate overnight at 37℃ on LB(Amp^+) screening plate.

2. Determination of the activity of carbonic anhydrase

A single colony from the above LB(Amp+) screening plate was cultured in 100mL LB(Amp+) liquid medium for 12h, and IPTG was added to induce expression for 12h.

Lysozyme-freeze-thaw method was used to extract crude enzyme solution. 10ml of bacterial solution was centrifuged at 5000rpm for 5min, and the supernatant was discarded. The bacterial cells were resuspended in 0.01m PBS buffer and centrifuged at 5000rpm for 5min to wash the bacterial cells. Add 5mL of 0.01M PBS buffer and 100 µl of 20mg/mL lysozyme solution and react at 4℃ for 30min. Freeze at -80℃ for 30min and then thaw at 4℃, repeat until the thawed liquid becomes sticky obviously. Centrifuge at 12000rpm for 10min, then collect supernatant for enzyme activity determination. Carbonic anhydrase activity was determined by p-NPA method.

The hydrolysate of p-nitrophenyl acetate (p-NPA) is p-nitrophenol, which can selectively absorb light at the wavelength of 348nm. Therefore, the enzyme activity can be calculated by measuring the absorbance value at this wavelength. The hydrolysis reaction was initiated by successively adding 5.4mL of fresh Tris buffer (100mM, pH7.6), 3mL of free enzyme, and 0.6mL of p-NPA dissolved in acetone (3mM) to a centrifuge tube. After 3min of reaction, the absorbance value at 348nm was measured by UV spectrophotometer, and the sample without enzyme was used as blank control. Relative enzyme activity was defined as the percentage of the ratio of any enzyme activity to the optimal enzyme activity under the same conditions.

3. Error prone PCR amplification

We use controlled error-prone PCR kit to perform error-prone PCR on mASCA conventional PCR products. In order to prevent the error-prone PCR effect from being unsatisfactory, the reaction systems with mASCA template concentrations of 1ng/µL and 10ng/µL were set as follows:

mASCA	1μL
Mix	3μL
dNTP	3μL
dGTP	2μL
$MnCl_2$	4μL
pR	1μL
pF	1μL

After predenaturation at 94℃ for 3min, 45 cycles of reaction were performed: denaturation at 94℃ for 1min, annealing at 45℃ for 1min, and extension at 72℃ for 1min. The error-prone PCR products are examined by electrophoresis on 1% agarose gel.

4. Construction and of mutation libraries

Error-prone PCR product and plasmid pETDuet-1 were purified by DNA purification kit and double digested with BamH Ⅲ and EcoR Ⅰ. The reaction system was as follows:

Gene	20 μL
10 × NEB	5 μL
BamHⅢ enzyme	1 μL
EcoRI enzyme	1 μL
ddH_2O	38 μL

The reaction was performed at 37℃ for 1h and then terminated at 80℃ for 20min. The digested products were recovered by DNA purification kit and quantified. Then, the two products were reacted with T4 DNA ligase at room temperature for 30min or overnight at 16℃, and then transformed into competent *E. coli* BL21(DE3) cells, and cultured overnight at 37℃ by LB(Amp^+) screening plate.

5. Determination of enzyme activity of recombinant enzymes

Steps are same as "Determination of the activity of carbonic anhydrase"

6. Bioinformatics analysis

We used primers pR/pF to PCR recombinant strains exhibiting higher enzyme activity, and the PCR products were sent to Sangon Biological Company for sequencing.

The gene sequence analysis and 3D structure prediction of mASCA was conducted with Phyer2 protein structure server. After finding the populations with high sequence similarity using Blastp server in NCBI, the sequences were aligned and the tree was built using the neighbor joining method in software Mega.

References

[1] KATARIA A, SHARMA R, SHARMA S, et al. Recent appli-cations of bio-engineering principles to modulate the functionality of proteins in food systems[J]. Trends in Food Science & Technology, 2021, 113: 54−65.
DOI: https://doi.org/10.1016/j.tifs.2021.04.055

[2] XU Y, WU Y, LV X, et al. Design and construction of novel biocatalyst for bioprocessing: Recent advances and future outlook [J]. Bioresource Technology, 2021: 125071.
DOI: https://doi.org/10.1016/j.biortech.2021.125071

[3] ZENG W, GUO L, XU S, et al. High-throughput screening technology in industrial biotechnology[J]. Trends in Biotechnolo-gy, 2020, 38 (8): 888−906.
DOI: https://doi.org/10.1016/j.tibtech.2020.01.001

[4] WU H, TIAN X, DONG Z, et al. Engineering of *Bacillus amyloliquefaciens* α-amylase with improved calcium independence and catalytic efficiency by error-prone PCR[J]. Starch-Stärke, 2018, 70 (3−4): 1700175.
DOI: https://doi.org/10.1002/star.201700175

[5] GAOL M D F L, SATYA A A, PUSPITASARI E, et al. In-creasing hydrolytic activity of lipase on palm oil by PCR-based ran-dom mutagenesis[J]. International Journal of Oil Palm, 2020, 3 (3): 78−87.
DOI: https://doi.org/10.35876/ijop.v3i3.53

[6] DAI S, YAO Q, YU G, et al. Biochemical characterization of a novel bacterial laccase and improvement of its efficiency by direct-ed evolution on dye degradation[J]. Frontiers in Microbiology, 2021: 12.
DOI: https://doi.org/10.3389/fmicb.2021.633004

[7] SUN Z B, XU J L, LU X, et al. Directed mutation of β-glu-canases from probiotics to enhance enzymatic activity, thermal and pH stability[J]. Archives of Microbiology, 2020, 202（7）: 1749−1756.
DOI: https://doi.org/10.1007/s00203-020-01886-z

[8] SU L, YAO K, WU J. Improved activity of Sulfolobus acido-caldarius maltooligosyltrehalose synthase through directed evolu-tion[J]. Journal of Agricultural and Food Chemistry, 2020, 68 (15): 4456−4463.
DOI: https://doi.org/10.1021/acs.jafc.0c00948

[9] BASIT A, TAJWAR R, SADAF S, et al. Improvement in ac-tivity of cellulase Cel12A of Thermotoga neapolitana by error prone PCR[J]. Journal of Biotechnology, 2019, 306: 118−124.
DOI: https://doi.org/10.1016/j.jbiotec.2019.09.011

[10] BOQUET E, BORONAT A, RAMOS – CORMENZANA A. Produc-tion of calcite (Calcium carbonate) crystals by soil bacteria is a gen-eral phenomenon [J]. Nature, 1973, 246(5434) : 527 – 529.
DOI: https://doi.org/10.1038/246527a0

[11] SHAHROKHI – SHAHRAKI R, O'KELLY B C, NIAZI A, et al. Improving sand with microbial – induced carbonate precipitation [J]. Proceedings of the Institution of Civil Engineers Ground Improve-ment, 2015, 168(3) : 1 – 14.
DOI: https://doi.org/10.1680/grim.14.00001

[12] WANG L, WANG XX, LI F, CUI MJ, YANG XX, YANG M, YAN YJ. Advances of enzymes related to microbial cement. Chin J. Biotech, 2022, 38(2): 506-517.
DOI: https://doi.org/10.13345/j.cjb.210127.

[13] Fujita M, Nakashima K, Achal V, et al. Whole-cell evaluation of urease activity of *Pararhodobacter sp.*isolated from peripheral beachrock. Biochem Eng J,2017, 124: 1-5.
DOI: https://doi.org/10.1016/j.bej.2017.04.004

[14] JUN S Y, KIM S H, KANTH BK, et al. Expression and characterization of a codon-optimized alkaline-stablecarbonic anhydrase from *Aliivibrio salmonicida* for CO2 sequestration applications[J/OL].Bioprocess and Biosystems Engineering, 2017, 40(3): 413-421.
DOI: https://doi.org/10.1007/s00449-016-1709-3.

[15] ACHAL V, PAN X.Characterization of Urease and Carbonic Anhydrase Producing Bacteria and TheirRole in Calcite Precipitation[J/OL]. Current Microbiology, 2011, 62(3):894-902.
DOI: https://doi.org/10.1007/s00284-010-9801-4.

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