



(51) International Patent Classification:

C12N 9/28 (2006.01) A21D 8/04 (2006.01)

(21) International Application Number:

PCT/CN2021/103084

(22) International Filing Date:

29 June 2021 (29.06.2021)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

202010876688.5 27 August 2020 (27.08.2020) CN

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(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ,

CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN,
KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD,
ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO,
NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW,
SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

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

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: A RECOMBINANT STRAIN PRODUCING MALTOGENIC AMYLASE AND ITS APPLICATION IN BAKED FOOD

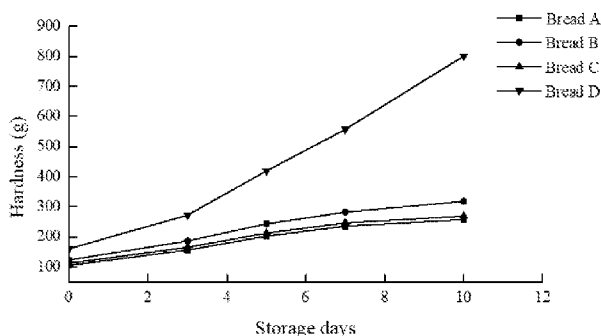


Fig. 1

(57) Abstract: Provided is a recombinant strain producing maltogenic amylase and its application in baked foods, belonging to the technical fields of genetic engineering, enzyme engineering and food engineering. The enzyme activity of recombinant maltogenic amylase BLMA is as high as 3235.0 U/mg at 60°C and pH 6.5, with good pH stability and wide range of temperature. Through the use of error-prone PCR, gene-directed mutagenesis technologies, mutant with better enzyme activity and better optimum temperature was obtained, the enzyme activity of the mutant BLMA1 was increased to 2.16 times, and the optimum temperature was increased to 65°C. By applying BLMA and BLMA1 to baked foods, both of them could effectively delay the aging of baked foods, and improve the shelf life of foods.



A Recombinant Strain Producing Maltogenic Amylase and its Application in Baked Food

CROSS-REFERENCES AND RELATED APPLICATIONS

[0001] This application claims priority of Chinese Application No. CN202010876688.5, entitled "A Recombinant Strain Producing Maltogenic Amylase and its Application in Baked Food", August 27, 2020, which is herein incorporated by reference in its entirety.

BACKGROUND

Field of the Invention

[0002] The present disclosure relates to recombinant strains producing maltogenic amylase and its application in baked foods, belonging to the technical fields of enzyme engineering and food engineering.

Description of the Related Art

[0003] Maltogenic amylase (EC 3.2.1.133), belonging to glycoside hydrolase family 13, is a type of endo-acting amylase that hydrolyzes the α -1,4-glucosidic bond. Maltogenic amylase enzymes can hydrolyze various substrates such as cyclodextrins, starches and pullulan to maltose, exhibiting multi-substrate specificity and catalytic versatility. Maltogenic amylase can be isolated from various sources, including *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus cereus*, *Thermus* sp., and *Thermus vulgaris*, etc. Different sources of maltogenic amylase have great differences in enzymatic properties.

[0004] During the storage of baked food, bread staling results in crumb hardening, crust softening and loss of the characteristic fresh flavor of the product, making the problem of bread staling one of the great challenges to the bread baking industry. Maltogenic amylase can hydrolyze starch to maltose, oligosaccharide and small molecule dextrin. These maltose and small molecule dextrin are too short to crystallize and form crystalline junction zones, interfere with the recrystallization of starch and the entanglement of starch granules and protein macromolecules, thereby reducing the regenerating rate and recrystallization rate of starch granules, keeping the bread soft and its freshness longer. At present, maltogenic amylase is gradually being used in the bakery industry, but high-quality and safe maltogenic amylase is rarely reported.

[0005] *B. subtilis* has strong protein exocrine ability and has no obvious codon preference. It has been recognized by the US Food and Drug Administration and China's food safety related departments as a food safety-grade strain GRAS (Generally recognized as safe). Therefore, it is a prefect expression strain for food-grade. But there exist problems such as low expression level and low recombinase activity by *B. subtilis* expressing maltogenic amylase. Therefore, the screening and construction of the recombinant maltogenic amylase in *B. subtilis* with high protein production, enzyme activity, excellent enzymatic properties are of great significance for the baked food.

DETAILED DESCRIPTION

[0006] To solve the above problems, the present disclosure screened a strain of *B. licheniformis* RZ108 that produces maltogenic amylase, used chromosome walking technology to clone the complete maltogenic amylase gene from its genomic DNA, and constructed an expression vector. The target gene was expressed in *B. subtilis* WB600, and a recombinant maltogenic amylase with high expression and high enzyme activity was obtained. The recombinant maltogenic amylase was applied to bread baking, the results showed it can improve the rheological properties of the dough, effectively delay the regeneration of starch, reduce the hardness of the bread during storage, and significantly improve the quality of the bread, thereby increasing the shelf life of the baking.

[0007] The present disclosure provides a maltogenic amylase with the amino acid sequence set forth in SEQ ID NO. 1.

[0008] The present disclosure provides a gene for coding the maltogenic amylase, and the nucleotide sequence set forth in SEQ ID NO.2.

[0009] The present disclosure provides a recombinant plasmid carrying the gene of maltogenic amylase.

[0010] In one embodiment of the present disclosure, the vector included but was not limited to pMA0911.

[0011] The present disclosure provides a host cell carrying the gene of maltogenic amylase.

[0012] The present disclosure provides a host cell carrying the recombinant plasmid.

[0013] The present disclosure provides a recombinant microbial cells expressing the maltogenic amylase.

[0014] In one embodiment of the present disclosure, the microorganisms included but were not limited to *B. subtilis* WB600.

[0015] In one embodiment of the present disclosure, the recombinant microorganism was a recombinant *B. subtilis*, taking pMA0911 as a vector to express the maltogenic amylase in *B. subtilis* WB600.

[0016] The present disclosure provides a recombinant *B. subtilis*, expressing the maltogenic amylase, and the recombinant *B. subtilis* takes pMA0911 as an expression vector.

[0017] The present disclosure provides a method for constructing the recombinant bacteria, which included the following steps: (1) ligating the maltogenic amylase gene to the vector pMA0911, (2) transforming the obtained recombinant expression vector into *B. subtilis* WB600 to obtain recombinant bacteria.

[0018] In one embodiment of the present disclosure, the complete maltogenic amylase gene was obtained by using chromosome walking technology.

[0019] In one embodiment of the present disclosure, restriction enzymes *EcoR* I and *Bam*H I were used to digest the target gene and plasmid.

[0020] In one embodiment of the present disclosure, lysozyme and a plasmid extraction kit were used to extract recombinant plasmids and to verify positive clones of recombinant bacteria.

[0021] In one embodiment of the present disclosure, *B. subtilis* was transformed by an improved Spizizen method.

[0022] The present disclosure provides a method for soluble expression of maltogenic amylase, which inoculated the recombinant bacteria into a culture medium and cultured it at 30-35°C for 24 to 48 h.

[0023] In one embodiment of the present disclosure, culturing the recombinant bacteria at 33 °C for 48 h.

[0024] In one embodiment of the present disclosure, the medium contains tryptone, yeast extract, glycerol, potassium dihydrogen phosphate, and dipotassium hydrogen phosphate.

[0025] In one embodiment of the present disclosure, the medium formula was 12 g/L tryptone, 24 g/L yeast extract, 5 g/L glycerol, 2.3 g/L potassium dihydrogen phosphate and 12.5 g/L potassium dihydrogen phosphate, pH 7.0.

[0026] In one embodiment of the present disclosure, an ÄKTA™ avant protein purification system was used for protein purification and DNS method was used to determine the enzyme activity.

[0027] The present disclosure provides an enzyme preparation, wherein it contains the pure maltogenic amylase or a mixture of the maltogenic amylase and an enzyme protective agent.

[0028] The present disclosure provides an application of the maltogenic amylase, or the recombinant *B. subtilis*, or the enzyme preparation, or recombinant microbial cells in the field of bakery products.

[0029] In one embodiment of the present disclosure, the baked products included but was not limited to baked bread.

[0030] In one embodiment of the present disclosure, the dough formula was 500 g flour, 35 g white sugar, 5 g edible salt, 5 g yeast, and 650 g water.

[0031] In one embodiment of the present disclosure, the bread baking formula was as follows: 1,000 g flour, 70 g white sugar, 10 g edible salt, 10 g yeast, and 650 g water.

[0032] The present disclosure provides a directed evolution method, which obtains a maltogenic amylase with better thermo-stability.

[0033] In one embodiment of the present disclosure, error-prone PCR technology was used to amplify the target gene and construct a mutant gene library, two rounds of screening were used to obtain ideal mutants, and the gene sequence of the obtained mutant was performed.

[0034] In one embodiment of the present disclosure, gene single-site-directed mutagenesis and multi-site-directed mutagenesis technology were used to obtain target mutants. The ideal mutants were obtained by comparing the enzyme activity and optimal temperature of all mutants.

[0035] The present disclosure provides a maltogenic amylase mutant, comprising an amino acid sequence with mutations of the 418th lysine and the 296th valine, wherein the mutations are relative to a parent amino acid sequence set forth in SEQ ID NO.1.

[0036] In one embodiment of the present disclosure, the maltogenic amylase mutant, comprising an amino acid sequence with mutations of the 418th lysine and the 296th valine, wherein the mutations are relative to a parent amino acid sequence set forth in SEQ ID NO.1. and the 296th position is mutated from valine to phenylalanine, the 418th position is mutated from lysine to isoleucine, the amino acid sequence of the maltogenic amylase mutant is set forth in SEQ ID NO.9.

[0037] The present disclosure provides a gene for coding the above-mentioned maltogenic amylase mutant.

[0038] The present disclosure provides a recombinant plasmid carrying the above-mentioned gene.

[0039] The present disclosure provides a recombinant microbial cell expressing the above-mentioned maltogenic amylase mutant.

[0040] The present disclosure provides a recombinant *B. subtilis*, expressing the maltogenic amylase mutant, and the recombinant *B. subtilis* takes pMA0911 as an expression vector.

[0041] The present disclosure provides an application of the above-mentioned mutant or the above-mentioned recombinant *B. subtilis* in the field of bakery products.

[0042] In one embodiment of the present disclosure, the bakery products include but is not limited to bread.

BENEFICIAL EFFECTS

[0043] The present disclosure constructed the recombinant strain *B. subtilis* WB600/pMA0911-BLMA, which could efficiently express the target gene, and applied it to baked food. The crude enzyme solution expressed by recombinant bacteria was purified by ammonium sulfate gradient sedimentation and His-Trap HP chromatography column to obtain pure recombinant enzyme BLMA. The optimum temperature of pure enzyme BLMA is 60 °C. In the temperature range of 30-80 °C, more than 60% of the enzyme activity is remained after 30 min incubation. The optimum pH is 6.5, over 60% of the enzyme activity can be retained in the range of pH 5.5-7.5. Under the conditions of 60 °C and pH 6.5, the activity of pure enzyme BLMA is as high as 3235.0 U/mg. The maltogenic amylase BLMA produced by the recombinant bacteria has the characteristics of high enzyme activity, good thermo-stability and a wide range of pH applications. After gene mutation, the enzyme activity of the most ideal mutant was increased to 2.16 times, and the optimum temperature was increased to 65 °C. By Applying BLMA and mutant to food baking, both of them can improve the rheological properties of the dough, effectively delay the regeneration of starch, reduce the hardness of the bread during storage, and increase the shelf life of baking. And 45 ppm mutant can reach litter better effect on

bread quality than 60 ppm BLMA, the application effect and economic value of mutant enzyme have been improved.

BRIEF DESCRIPTION OF FIGURES

- [0044] Fig. 1 The hardness of bread during storage.
- [0045] Fig. 2 The elasticity of bread during storage.
- [0046] Fig. 3 The activity and optimal temperature of enzyme for single-site-directed mutagenesis.
- [0047] Fig. 4 Enzyme activity of double site-directed mutants.
- [0048] Fig. 5 Optimum temperature of maltogenic amylase produced by strains 5-8.
- [0049] Fig. 6 The thermostability of maltogenic amylase produced by strains 5-8.
- [0050] Fig. 7 The hardness and elasticity of bread during storage.
- [0051] Fig. 8 The sensory score of bread.

EXAMPLES

Example 1: Isolation of maltogenic amylase-producing strain

[0052] Samples: Soils with rich starch were collected from Wu-xi County, Jiangsu Province, P. R. China.

[0053] Liquid medium: 5 g/L NaCl, 0.1 g/L MgSO₄, 0.5 g/L KH₂PO₄, 0.2 g/L CaCl₂, 0.3 g/L yeast extract, 0.3 g/L tryptone, pH 6.0.

[0054] Plate medium: 0.2% soluble starch, 1% protein, 0.5% NaCl, 0.3% beef extract, 2% agar, pH 7.0.

[0055] Lugol's solution: 0.3% I₂, 0.6% KI.

[0056] Isolation method: Soil samples with rich starch were cultured in liquid medium and incubated at 37 °C, with 200 rpm shaking for 24 h. 1 mL of culture solution was taken and diluted with physiological saline (1×10^{-1} , 1×10^{-2} , 1×10^{-3} , 1×10^{-4} , 1×10^{-5}), the diluent was spread on the screening plate, and cultured at 30 °C for 56 h. Lugol's solution was added to the plate at 20 °C, and the clones with the prevalent hydrolysis circle were selected. The first screened strains were inoculated in LB liquid medium, and cultivated at 30 °C, 200 rpm for 12 h. 3% of the inoculum was inoculated at 50 mL enzyme production medium, cultivated at 30 °C, 200 rpm for 48 h, and placed in a refrigerated centrifuge at 8,000 rpm, centrifuged for 5 min, and then took the fermentation supernatant to determine the enzyme activity.

Example 2: Identification of strains producing maltogenic amylase

[0057] According to Bergey's manual, the isolate was identified using bacteriological tests. The morphological, physiological and chemical properties of the strain were analyzed, and the strain was initially identified as *Bacillus* sp. By constructing a phylogenetic tree and comparing the similarity of 16S rRNA sequence, the strain was determined to be *B. licheniformis*, named *B. licheniformis* RZ108.

Example 3: Analysis of partial amino acid sequence of maltogenic amylase

[0058] The protein produced by *B. licheniformis* RZ108 was analyzed by SDS-PAGE, and the protein band produced by maltogenic amylase was cut and digested with trypsin. The released short peptide sequence was analyzed by MALDI-TOF-MS to obtain the partial amino acid sequence of maltogenic amylase.

Example 4: Cloning and sequence analysis of maltogenic amylase gene

[0059] Primer synthesis: P1: AARWSNAARTGGAARATGT (SEQ ID NO.3) ; P2: NTTYACNGTNAARWSNGCNC (SEQ ID NO.4) ; P3: TTTGACGGCGTCGATGC (SEQ ID NO.5) ; P4: GCGAATGGTATGGCG (SEQ ID NO.6) ; P5: TTCCAGATGGTTGGCCGT (SEQ ID NO.7) ; P6: TGAGCGTCAACAGCAACA (SEQ ID NO.8) .

[0060] PCR conditions: 98 °C thermal denaturation 30 s; 98 °C 15 s, 55 °C 15 s, 72 °C 2 min; after 30 cycles, 72 °C extends for another 5 min.

[0061] The degenerate primers P1 and P2 were designed to amplify part of the sequence of the gene with *B. licheniformis* RZ108 genomic DNA as template and P1 and P2 as primers. To obtain the complete gene sequence, two rounds of inverse PCR were applied to characterize the flanking sequences of the known fragments. First, the genomic DNA of *B. licheniformis* RZ108 was digested with *Sma* I, following by the self-ligation under conditions conducive to form the monomer loops by T4 DNA ligase. The first round of inverse PCR was performed with primers P3 and P4 to amplify the flanking fragment (0.96 kb). The second round of inverse PCR was performed using the RZ108 genomic DNA digested by *Nco* I as template, with primers P5 and P6, to amplify the flanking fragment (1.1 kb). Using Snap Gene 2.3.2 to analyze the gene fragments, the possible start codon ATG is located in the 0.96 kb fragment, the possible stop codon TAA is located in the 1.1 kb fragment, and the catalytic triad Asp-Glu-Asp is located in the 1.1 kb fragment. Assemble the three fragments to obtain the complete coding gene BLMA (1,707 bp).

Example 5: Construction of recombinant plasmid pMA0911-BLMA

[0062] The plasmid pMA0911 was extracted using the Mini-Plasmid Rapid Isolation Kit (Beijing BioDev-Tech. Co., Ltd). The water, buffer, plasmid pMA0911 and enzyme were added by order to the Eppendorf tube, and the tube was shaken to mix the liquid thoroughly. The tube was placed in a centrifuge for 2 s to concentrate the liquid on the bottom of the tube, incubated at 37 °C for 1 h. Then the tube was kept at 65 °C for 10 min to terminate the digestion reaction. The digested products were analyzed by agarose gel electrophoresis, and the gel was recovered and concentrated.

[0063] Enzyme digestion system: 30 µL plasmid, 13 µL sterile water, 5 µL 10×quick-cut enzyme buffer, 1 µL restriction endonuclease *EcoR* I, 1 µL restriction endonuclease *BamH* I.

[0064] The reaction system of connection between the target gene and the plasmid T-BLMA and PMA0911: 0.5 µL T4 DNA Ligase, 1 µL 10×T4 DNA Ligase buffer, plasmid and gene in a molar ratio of 1:7, total 8 µL. Put the mixed connection solution in a 16 °C incubator and connect for 30 min.

Example 6: Construction of recombinant *B. subtilis* WB600/pMA0911-BLMA

[0065] 10 µL of the ligation product was added to 500 µL of *B. subtilis* WB600 competent cell suspension, mixed the mixture gently at 37 °C, and placed for 30 min with 100 rpm shaking. Then the centrifuge tube was placed in a shaker at 37 °C, 250 rpm shaking, for 1.5 h. After the culture was completed, the bacteria was collected by centrifugation at a shaking with 4,000 rpm, the supernatant was discard, 100 µL to resuspend the bacteria was leaved and spread on the LB plate containing 50 µg/mL kanamycin sulfate, finally, the culture was inverted overnight at 37 °C.

[0066] Selection of positive clones: 4 clones was picked from each plate and transferred to 5 mL of LB medium containing the corresponding antibiotics and incubated at 37 °C for 8 h. The lysozyme and plasmid extraction kit Mini- Plasmid Rapid Isolation Kit (Beijing BioDev-Tech. Co., Ltd) was used to extract plasmids. The restriction digestion was verified by using the reaction system containing: 2 µL 10×Buffer H, 5 µL DNA, 0.5 µL *EcoR* I, 0.5 µL *Xho* I, adding H₂O to make up the system to 20 µL. The positive plasmid pMA0911-BLMA was obtained by DNA sequencing verification.

Example 7: Expression and purification of maltogenic amylase

[0067] TB medium: 12 g/L tryptone, 24 g/L yeast extract, 5 g/L glycerol, 2.3 g/L potassium dihydrogen phosphate, 12.5 g/L dipotassium hydrogen phosphate, 50 µg/mL kanamycin sulfate, pH 7.0.

[0068] The positive clone *B. subtilis* WB600/pMA0911-BLMA was picked and then placed in 20 mL TB liquid medium and cultured at 33°C for 48 h.

[0069] The cultured recombinant *B. subtilis* cells were centrifuged at 4 °C, with 8,000 rpm shaking for 15 min to remove the bacteria, and the fermentation supernatant was collected.

[0070] Ammonium sulfate of 30% was slowly added to the supernatant. After 4 h at 4 °C, the supernatant was centrifuged with 8,000×g shaking at 4 °C for 10 min to remove the contaminated proteins. A portion of ammonium sulfate powder was added to the supernatant to make the final concentration of ammonium sulfate in the supernatant 50%. After standing at 4 °C for 6 h, the supernatant was centrifuged at 10,000×g at 4 °C for 30 min to collect the precipitate.

[0071] The precipitate was reconstituted with 20 mM Tris-HCl buffer (pH 8.0), and then further purified using ÄKTA™avant protein purification system, and the solution was loaded onto a HisTrap HP column (GE Healthcare, Chicago, USA). The column was eluted with 20 mM phosphate buffer (pH 8.0) containing 20 mM imidazole and 500 mM NaCl, and the collected target protein solution (about 2 mL) was concentrated with an ultrafiltration tube (Millipore Amicon Ultra, Billerica, USA). The imidazole was removed from the column by using desalting, and the protein purity was judged by SDS-PAGE analysis.

Example 8: Determination of Maltogenic Amylase Activity

[0072] (1) Principle and definition: Maltogenic amylase can hydrolyze starch into reducing sugars, and reducing sugars can form brown-red amino complexes with 3,5-dinitrosalicylic acid under heating conditions. Within a certain range, the amount of reducing sugars and the color of the reaction solution is directly proportional. A spectrophotometer can be used for colorimetric measurement at 540 nm to obtain the sugar content in the sample.

[0073] (2) Standard conditions for enzyme activity determination: In 50 mM phosphate buffer (pH 6.0), taking 500 µL 1% soluble starch solution and place it in a 2 mL eppendorf tube, preheat it at 60 °C for 10 min. Adding 100 µL diluted enzyme solution, mix well, after reacting for 10 min, add 600 µL DNS to stop the reaction. Taking 200 µL of the solution into a 96 shallow well plate. Use a multifunctional microplate reader to measure the absorbance at a wavelength of 540 nm. The protein content is determined by Bradford method, with BSA as the

standard. One unit of maltogenic amylase was defined as the amount of enzyme that released reducing sugars equivalent to 1 mg of glucose per minute under the above condition.

[0074] (3) Calculation formula for enzyme activity: $U = n \times A \times K / 10$; Enzyme activity (U/mg) = $U / \text{protein content (mg)}$; n -dilution multiple; A -the average absorbance of experiment; K -absorption constant; 10-reaction time(10min).

Example 9: Determination of optimum temperature

[0075] The enzyme was incubated in phosphate buffer (pH 6.5) under a temperature gradient of 40 to 90 °C. The enzyme activity of maltogenic amylase BLMA at different temperatures was calculated and summarized in Table 1. The highest enzyme activity is 3235.0 U/mg, in the range of 55-75 °C, the enzyme activity can still retain more than 75% of the total activity.

Table 1 Enzyme activity of BLMA at different temperature

Temperature (°C)	Enzyme activity (U/mg)
40	1191.8
45	1879.8
50	2186.9
55	3042.0
60	3235.0
65	3101.7
70	2923.2
75	2514.3
80	2077.8
85	1273.2
90	239.5

Example 10: Determination of optimum pH

[0076] The optimum pH of BLMA was determined in buffers with different pH: 50 mM citrate buffer (pH 4.0-6.0), 50 mM phosphate buffer (pH 6.0-7.0) and 50 mM Tris-HCl buffer (pH 7.0-9.0), the measurement temperature is 60 °C. The enzyme activity of BLMA measured at different pH was shown in Table 2. In the pH range of 5.0-8.0, the enzyme activity of the recombinase reaches more than 80% of the total activity, indicating that BLMA has a wider pH adaptation range.

Table 2 Enzyme activity of BLMA at different pH

pH	Enzyme activity (U/mg)
4.0	672.4
4.5	1684.7
5.0	2630.0
5.5	2818.4
6.0	3078.8

6.5	3235.0
7.0	2954.3
7.5	2808.0
8.0	2625.9
8.5	1791.1
9.0	1274.3

Example 11: Determination of thermo-stability

[0077] After incubating the maltogenic amylase BLMA at different temperatures (30-90 °C) for 30 min, the remaining enzyme activity was detected, and the untreated enzyme activity was defined as 100%. The remaining enzyme activity data after incubation at different temperatures was shown in Table 3. Recombinant BLMA could tolerate temperature from 30 °C to 80 °C, and still retained more than 60% of the enzyme activity after 30 min of incubation, indicating that BLMA can work between 60 °C and 80 °C. When BLMA acts at 60-80 °C, the enzyme activity can be maintained for a long time, the catalytic effect will be better. During bread baking, the temperature of the bread can reach above 95 °C, while after BLMA is treated at 90 °C for 30 min, the residual enzyme activity is 0, which means BLMA will not cause the baked product to sticky. BLMA's wide application temperature range and good temperature stability provide huge potential advantages for its application in the bread baking industry.

Table 3 Remaining enzyme activity of BLMA after incubated at different temperature for 30 min

Temperature (°C)	Remaining enzyme activity (%)
30	100
40	97
50	90
60	83
70	75
80	60
90	0

Remarks: When measuring the thermo-stability of BLMA, the pH of the buffer used is 6.5.

Example 12: Determination of pH stability

[0078] The remaining enzyme activity of recombinant maltogenic amylase BLMA in buffers of different pH (50 mM citrate buffer, pH 4.0-6.0, 50 mM phosphate buffer, pH 6.0-7.0 and 50 mM Tris-HCl buffer, pH 7.0-9.0) after 30 min of incubation was detected. The enzyme activity at pH 6.5 was taken as 100%. The remaining enzyme activity at different pH was shown in Table 4. Recombinant BLMA showed good stability in the pH range of 5.5-7.5, and its enzyme activity remained above 60%.

Table 4 Remaining enzyme activity of BLMA after incubated at different pH for 30 min

pH	Remaining enzyme activity (%)
4.0	12
4.5	20
5.0	58
5.5	69
6.0	75
6.0	80
6.5	100
7.0	86
7.0	79
7.5	68
8.0	29
8.5	20
9.0	14

Remarks: When determining the pH stability of BLMA, the temperature used is 60 °C.

Example 13: Determination of ionic stability

[0079] Put the recombinant maltogenic amylase BLMA in a solution (pH 6.5) containing 5 mM metal ions (Mn^{2+} , Li^+ , Ca^{2+} , Ba^{2+} , Mg^{2+} , Co^{2+} , K^+ , Cu^{2+} , Fe^{2+} and Zn^{2+}), and chemical substances (EDTA and SDS). After incubating the solution at 60 °C for 30 min, the remaining enzyme activity of BLMA was detected to determine the ion dependence of the enzyme and the influence of chemical substances. Taking the BLMA enzyme activity without adding any metal ions or chemicals as 100%, the relative enzyme activity of recombinant BLMA was shown in Table 5. Mn^{2+} , Ba^{2+} , Cu^{2+} , Fe^{2+} , Zn^{2+} , EDTA and SDS inhibited the activity of enzyme, while the presence of Ca^{2+} improved enzyme activity, suggesting BLMA had strong Ca^{2+} dependence.

Table 5 Relative enzyme activity of BLMA after incubated in different ionic solutions for 30 min

Metal ions and chemicals	Relative enzyme activity (%)
Control	100 ± 3.94
Mn^{2+}	73.26 ± 2.72
Li^+	91.64 ± 2.58
Ca^{2+}	186.37 ± 3.56
Ba^{2+}	79.42 ± 1.92
Mg^{2+}	99.38 ± 3.89
Co^{2+}	103.26 ± 2.43
K^+	106.67 ± 4.25
Cu^{2+}	30.81 ± 1.24
Fe^{2+}	59.71 ± 1.68
Zn^{2+}	56.92 ± 2.28
EDTA	32.64 ± 1.25
SDS	2.51 ± 0.12

Example 14: Determination of rheological properties of dough

[0080] Dough formula: 500 g flour, 35 g white sugar, 5 g edible salt, 5 g yeast, and 650 g water.

[0081] Method: AACC 54-60 (AACC, 2000) standard was used to test the dough rheology and the Mixolab (Chopin Technologies, France) was used to determine the properties of the dough, following "Chopin+" Standard setting test conditions: water absorption rate of 58%, hydration rate reference of 14%, dough speed of 80 rpm, target torque of (1.1 ± 0.5) Nm, dough weight of 75 g. The temperature control includes three phases: (1) constant temperature phase: 30 °C for 8 min; (2) heating phase: within 15 min, the temperature is raised to 90 °C at 4 °C/min and maintained at high temperature for 7 min; (3) cooling stage: the temperature is reduced to 50 °C at a rate of 4 °C/min within 10 min, and the temperature is maintained for 5 min.

[0082] The effects of maltogenic amylase BLMA (3235.0 U/mg) on the dough quality were investigated by adding different amounts of enzyme (0, 30, 60, and 90 ppm) to the dough. The sample without treatment of enzyme was used as control. The water absorption rate of the dough was measured first, according to the water absorption rate, an appropriate amount of maltogenic amylase was added for the experiment, and finally C1, C2, C3, C4 and C5, as well as the formation time, stability time and other parameters were shown in Table 6.

Table 6 The characteristics of the dough with the recombinant BLMA

BLMA	0 ppm	30 ppm	60 ppm	90 ppm
C1 (Nm)	1.16±0.01 ^b	1.18±0.01 ^b	1.21±0.01 ^a	1.20±0.02 ^a
C2 (Nm)	0.69±0.01 ^c	0.75±0.01 ^b	0.79±0.01 ^a	0.78±0.02 ^a
T1 (min)	9.80±0.05 ^a	9.40±0.05 ^b	9.20±0.05 ^c	9.20±0.05 ^c
Ts (min)	11.80±0.12 ^b	12.10±0.16 ^{ab}	12.40±0.12 ^a	12.20±0.12 ^a
C1-C2	0.47±0.02 ^a	0.43±0.01 ^b	0.42±0.01 ^b	0.42±0.01 ^b
C3 (Nm)	1.74±0.03 ^a	1.79±0.02 ^a	1.82±0.03 ^a	1.80±0.02 ^a
C4 (Nm)	1.52±0.02 ^a	1.41±0.02 ^b	1.36±0.01 ^c	1.20±0.02 ^d
C5 (Nm)	3.64±0.03 ^a	2.81±0.03 ^b	2.62±0.02 ^c	2.48±0.04 ^d
C3-C4	0.22±0.01 ^a	0.38±0.02 ^b	0.46±0.04 ^b	0.60±0.01 ^c
C5-C4	2.12±0.05 ^a	1.40±0.04 ^b	1.26±0.02 ^{bc}	1.28±0.06 ^c

Remarks: C1: maximum dough consistency; C2: protein weakening under mechanical forces and temperature degree; T1: the dough formation time; Ts: the stability time that the torque produced is kept at 1.1 Nm; C1-C2: total weakening value; C3: the gelatinization of starch during heating; C4: the stability of starch gelatinized adhesive; C5: the starch retrogradation during the cooling stage; C3-C4: starch breakdown; C5-C4: starch retrogradation at cooling stage. Data are expressed as the means ± standard deviations (n = 3). Values followed by different lower-case letters in the same row are significantly different from each other (p < 0.05).

[0083] The amount of recombinant BLMA had a slight impact on the formation time. However, the stability time T_s of the dough has increased, because the longer the stability time of the dough, the better the toughness, quality and applicability of the dough, therefore, adding 60 ppm of BLMA is beneficial to provide dough strength and mixing endurance. The peak viscosity of the dough C3 didn't change much, but there were significant differences in the viscosity disintegration value C3-C4 and the retrogradation value C5-C4 ($p < 0.05$). The C5-C4 value was lower, the retrogradation of starch was lower. The dough made using 60 ppm BLMA exhibited the lowest value of C5-C4 (1.26), adding the recombinant enzyme at 60 ppm effectively reduces the retrogradation of starch.

Example 15: Application of Maltogenic Amylase in Bread Baking

[0084] Bread formula: 1,000 g flour, 70 g white sugar, 10 g edible salt, 10 g yeast, and 650 g water.

[0085] Baking bread process: First, all the ingredients were put into the dough mixer (Hauswirt, Qingdao, China). The dough was kneaded at a low speed for 4 min, and at a high speed for 2 min. Put the dough into the SM-40SP proofer (Xinmai Machinery Co. Ltd China) for 40 min (30 °C, 85% RH, first proof). After the dough was relaxed for 5 min, it was divided into pieces and proofed for 30 min (30 °C, 85% RH, second proof). The final dough temperature was about 26-27 °C. Next, the dough from the container was removed, and was covered with a thin, clean plastic, and kept for about 5 min. Finally, the bread was baked in a Z202 oven (Demax Technology Co., Ltd. Foshan, China) at temperature of 170 °C. The baking time was 32 min. After freshly baked bread was cooled for 1 h, it was placed in a polyethylene bag and placed at 25 °C and 33% RH.

[0086] Bread with maltogenic amylase: Three kinds of maltogenic amylase were added for bread baking: (A) 60 ppm (BLMA, 3235.0 U/mg), (B) 90 ppm (MAM100 provided by Angel Yeast Company, 2438.0 U/mg), (C) 60 ppm (Novamyl 3D BG from Novozymes Investment Co. Ltd, 3073.0 U/mg). The bread without treatment of maltogenic amylase was used as a control group D.

[0087] Determination of bread specific volume: The specific volume of bread reflects the proofing and holding power of the dough: the larger the specific volume of bread, the easier the bread is to proof. It was measured according to the method described in AACC, 10-05 (AACC, 2000). Specific volume (SV) was calculated as the ratio bread apparent volume (V) and mass (m).

The specific volume of the four groups of bread was shown in Table 7. The specific volumes of bread in groups A (60 ppm BLMA), B (90 ppm MAM100) and C (60 ppm Novamyl 3D BG) were significantly higher ($p < 0.05$) than those of the control group D by 32.0%, 24.7%, and 30.3%, respectively. The maltogenic amylase hydrolyzed the starch to maltose during the dough fermentation. The maltose was then hydrolyzed to produce glucose by maltase from the baker's yeast, thus accelerating the proliferation of the yeast, and increasing gas production thus improving the specific volume of the bread.

Table 7 The specific volume of bread

Bread	The specific volume of bread (mL/g)
A	7.01 ± 0.04^a
B	6.62 ± 0.09^b
C	6.92 ± 0.07^a
D	5.31 ± 0.06^c

Remarks: Specific volumes of bread are expressed as the means $\pm$ standard deviations ($n = 3$). Different letters represent significant differences ($p < 0.05$).

[0088] Determination of bread hardness: The hardness of bread was evaluated by a texture analyzer TA-XT 2 (Shanghai BosinTech Co. Ltd) according to AACC74-09 (AACC, 2000). After the breads were stored for 0, 3, 5, 7 and 10 days, they were cut into 12.5 mm thick slices from the center. The two slices were stacked together as a sample for the test of their hardness and elasticity. Each 2 cm thick slice was fixed on the stage and compressed to 50% of the height using a cylindrical aluminum probe with a diameter of 25 mm. Then the trigger force was set to 5 g, the pretest speed was 1 mm/s, the test speed, was 1 mm/s, the post-test was 5 mm/s, and the gap between compressions was 30 s. The hardness of bread was calculated from force-distance diagrams. The effect of BLMA on bread hardness was shown in Figure 1. Compared with the control group D, the bread hardness of groups A, B and C with added maltogenic amylase was significantly reduced by 2.12, 1.52 and 1.98 times, respectively, after 10 days of storage. Group A with the recombinant maltogenic amylase BLMA of the present disclosure had a better hardness during storage.

[0089] Determination of bread elasticity: The elasticity of bread was calculated the same method as hardness. The effect of BLMA on bread elasticity was shown in Figure 2. Compared with the control group D, BLMA can significantly inhibit the decrease of bread elasticity after 3, 5, 7 and 10 days of storage, indicating that the maltogenic amylase BLMA can effectively delay the retrogradation of starch and increase the baking shelf life.

[0090] Sensory evaluation of bread: On the 7th day of storage, three kinds of bread with the treatment of different maltogenic amylase were randomly sampled and used for sensory evaluation. The sensory evaluation of bread was based on the method of Patil, which was performed by 20 trained sensory panel members using a 9-point hedonistic scale (like extremely, like very much, like moderately, like slightly, neither like nor dislike, dislike slightly, dislike moderately, dislike very much, dislike extremely). The sensory attributes appearance, color, smoothness, texture, flavor, taste, stomata and overall acceptability were evaluated with bread, and the scores are shown in Table 8. Bread A with BLMA was superior to the blank group bread D in all parameters, and the total score of bread A was the highest compared to bread B and bread C, indicating that the recombinant maltogenic amylase BLMA of the present disclosure was beneficial to maintain the taste, texture, and flavor of bread during storage, and can provide a longer shelf life for the finished product.

Table 8 Sensory score of bread

Bread	A	B	C	D
Appearance	7.5	6.8	7.8	6.2
Color	6.8	6.2	7.2	5.1
Smoothness	7.6	6.9	7.3	5.2
Texture	7.8	6.8	7.5	3.8
Flavor	7.3	7.5	6.9	4.2
Taste	7.0	5.1	6.4	2.2
Stomata	6.5	5.5	6.9	5.0
Overall Acceptability	7.3	6.2	7.0	4.6
Total Score	57.8	51.0	57.0	36.3

Example 16: Directed Evolution of Maltogenic Amylase

[0091] Directed evolution method: Error-prone PCR technology was used to amplify the target gene. When using DNA polymerase to amplify the target gene, by artificially adjusting the reaction conditions, such as increasing the concentration of Mn^{2+} , changing the concentration of the four dNTPs in the system, or using low-fidelity DNA polymerase to change the amplification, so as to randomly introduce mutations into the target gene at a certain frequency to obtain random mutants of protein molecules.

[0092] PCR conditions: 94 °C thermal denaturation 10 s; 98 °C 10 s, 55 °C 5 s, 72 °C 2 min; after 30 cycles, 72 °C extends for another 5 min.

[0093] Primers synthesis: F: TTTAAGAATTCATGCGCAAAGAAGCCATCCATCATC (SEQ ID NO.10) ;

R: TTTAAGGATCCTTACTGGATCGTTTTGCCGGCCG (SEQ ID NO.11) .

[0094] Reaction system:

Table 9 The reaction system of error-prone PCR

Composition	Volume (μ L)
10x rTaq buffer	5.0
25 mM $MgCl_2$	5.0
1-10 mM $MnCl_2$	5.0
dNTP (each 2.5 mM)	4.0
Primers F	0.5
Primers R	0.5
DNA	0.5
rTaq DNA polymerase (5U/ μ L)	0.5
ddH ₂ O	29.0

[0095] Single-factor experiment: The error-prone PCR conditions were explored by adjusting the concentration of Mn^{2+} in the reaction system, as shown in Table 10.

Table 10 The concentration of Mn^{2+} in the reaction system

Number	1	2	3	4	5	6	7	8	9	10
Mn^{2+} (mM)	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0

[0096] Screening of mutants: The gene expression vector was constructed to obtain the gene mutation library, and the mutation library was screened by measuring the enzyme activity of the crude enzyme solution. By comparing the enzyme activity of mutants and BLMA, the mutants with higher enzyme activity were obtained in the preliminary screening; the optimum temperature of the preliminary screening mutants was measured, and the mutants with higher optimum temperature were obtained in the second screening. Through screening, two strains with significantly improved enzyme activity or optimum temperature were obtained, named F1 and F2, respectively, as shown in Table 11. Taking the enzyme activity of BLMA before mutation as 100%, its optimum temperature is 60 °C. After the mutation, the optimum temperature of the F1 mutant was increased to 65 °C, the enzyme activity of F2 has greatly increased to 1.84 times.

Table 11 Mutants of error-prone PCR

Mutants	Relative enzyme activity (%)	Optimum temperature (°C)
F1	142	65
F2	184	60

[0097] Determination of the mutation sites: The mutants F1 and F2 were sequenced and identified. The sequencing results of F1 and F2 were compared and analyzed with the original gene of BLMA, a total of 8 gene mutation sites were discovered, as shown in Table 12.

Table 12 The mutation sites of error-prone PCR

Mutants	Mutation sites
F1	A51T、D218Q、V296F、T401I、K418I
F2	S110V、T267E、E397K

[0098] Mutants with one mutation site: Under the premise of known mutation sites, PCR-mediated site-directed mutagenesis technology was used to obtain eight mutants with only one mutation site, the mutants are numbered 1-8 according to the amino acid sequence. Using BLMA-pMA0911 as a template, single-point mutations were performed using the whole plasmid PCR method. The primer design is shown in Table 13 (the mutation site is shown as the underline).

Table 13 The primer sequence of single-site-directed mutagenesis

Mutation sites	Primer (5'-3')
1.A51T	tctgaa <u>act</u> gccagggtgccgtcatgccattc (SEQ ID NO.12) gaatggcatgacggcacctggcagtttcaga (SEQ ID NO.13)
2.S110V	gccgtccagctgttcgcccatggcg (SEQ ID NO.14) gcagggtggctatgttgctgttgacgtcagaa (SEQ ID NO.15)
3.D218Q	ccttcggcaaaatagctctgggtggctttaaaccggcg (SEQ ID NO.16) cgccgtttaaagccaaccagagctatittgccgaagg (SEQ ID NO.17)
4.T267E	cggccgggtcctcaaaagttttccactgggcttc (SEQ ID NO.18) gaagcccagtggaataaactttgaggaccggcg (SEQ ID NO.19)
5.V296F	aacgtgatcagggttttttatatttatctggtgccgtctgg (SEQ ID NO. 20) cgccatgggccaacagctggacggc (SEQ ID NO.21)
6.E397K	cggccgggtcctcaaaagttttccactgggcttc (SEQ ID NO.22) cgccgtttaaagccaaccagagctatittgccgaagg (SEQ ID NO.23)
7.T401I	ccagacgggcaacgaatataaataaaaaaacctgatcacgtt (SEQ ID NO.24) atataaataaagaaaacctgatcatattatcgacaaccatgac (SEQ ID NO.25)
8.K418I	ttctgagcgtcaacagcaacatagccaacctgc (SEQ ID NO.26) aacaacaacccggccaagtcctatggcctgtatgaccc (SEQ ID NO.27)

[0099] PCR conditions: 98 °C thermal denaturation 30 s; 98 °C 15 s, 55 °C 15 s, 72 °C 2 min; after 30 cycles, 72 °C extends for another 5 min.

[00100] Reaction system: 25 μL PrimeSTAR, 22 μL ddH₂O, 1 μL Primers F, 1 μL Primers R, 1 μL DNA.

[00101] The qualified mutants were screened by measuring the enzyme activity and optimum temperature of the mutants. Taking the enzyme activity of BLMA as 100%, the enzyme

activity and optimum temperature of mutants were shown in Figure 3. The enzyme activity was not improved better than F2 and the optimum temperature was not increased higher than F1.

[00102] Mutants with two mutation sites: The random combination method was used to determine the mutation site combination, and the multi-site-directed mutagenesis technology was used to obtain mutants with two mutation sites. The mutants that meet requirements were screened by measuring the enzyme activity and temperature of the mutants. Taking the enzyme activity of BLMA as 100%, the enzyme activity was shown in Figure 4, The enzyme activity of strains 5-8 was increased to 2.16 times that of BLMA. The optimum temperature and thermo-stability of the mutant strain 5-8 were shown in Figure 5 and Figure 6. The optimum temperature was higher than that of BLMA, and retained more than 75% of the total activity in the range of 55 °C to 80 °C. After 30 min of treatment at 60 °C to 80 °C, the residual enzyme activity was 60%, after 30 min of treatment at 85 °C, the remaining enzyme activity was 33%. Compared with BLMA, the operating temperature range has been expanded. And after being treated at 90 °C for 30 min, the residual enzyme activity of the strain 5-8 is 0, which would not have a negative impact on bread quality due to its non-inactivation under high temperature conditions. The maltogenic amylase produced by this mutant strain 5-8 was named BLMA1, the BLMA1 maltogenic amylase mutant, comprising an amino acid sequence with mutations of the 418th lysine and the 296th valine, wherein the mutations are relative to a parent amino acid sequence set forth in SEQ ID NO.1, and the 296th position is mutated from valine to phenylalanine, the 418th position is mutated from lysine to isoleucine. The amino acid sequence of the maltogenic amylase mutant BLMA1 is set forth in SEQ ID NO.9.

[00103] Application of BLMA1 on dough making: 60 ppm, 45 ppm and 30 ppm mutant BLMA1 (6987.6 U/mg) were added to the dough, the dough formula and test method were the same as example 14. The characteristics of the dough were shown in Table 14. There was significant difference on the value of C3-C4 and value of C5-C4 between the dough with 60 ppm BLMA1 and the dough with 60ppm BLMA (p <0.05). In the viscosity stage, the higher activity of mutant BLMA1 reduced the structural rigidity of starch granules, when the dough cooled, mutant BLMA1 was more conducive to maintaining dough elasticity, reducing the retrogradation of starch. Among the doughs with BLMA1 (30 ppm, 45 ppm, 60 ppm), the dough with 30 ppm BLMA1 showed the worst effect, and there was no big difference between the results of 45 ppm BLMA1 and 60ppm BLMA (p >0.05), which mean adding 45 ppm of BLMA1 could achieve

ideal effect. Using mutant BLMA1 to treat dough not only obtained good results but also reduced the amount of maltogenic amylase, compared with BLMA, its economic benefits were more prominent.

Table 14 The characteristics of dough

Characteristics	60 ppm BLMA	30 ppm BLMA1	45 ppm BLMA1	60 ppm BLMA1
C1 (Nm)	1.21±0.01 ^a	1.20±0.02 ^a	1.22±0.02 ^a	1.23±0.04 ^a
C2 (Nm)	0.79±0.01 ^a	0.77±0.02 ^b	0.81±0.01 ^a	0.82±0.01 ^a
T1 (min)	9.20±0.05 ^a	9.30±0.08 ^a	9.20±0.08 ^a	9.10±0.05 ^a
Ts (min)	12.40±0.12 ^a	12.20±0.12 ^a	12.40±0.09 ^a	12.50±0.09 ^a
C1-C2	0.42±0.01 ^a	0.43±0.02 ^a	0.41±0.03 ^a	0.41±0.04 ^a
C3 (Nm)	1.82±0.03 ^a	1.80±0.04 ^a	1.83±0.05 ^a	1.84±0.05 ^a
C4 (Nm)	1.36±0.01 ^a	1.38±0.02 ^b	1.35±0.02 ^a	1.33±0.01 ^{ab}
C5 (Nm)	2.62±0.01 ^b	2.74±0.05 ^a	2.57±0.03 ^{bc}	2.49±0.05 ^c
C3-C4	0.46±0.04 ^a	0.42±0.05 ^a	0.48±0.06 ^{ab}	0.51±0.06 ^b
C5-C4	1.26±0.02 ^b	1.36±0.03 ^a	1.22±0.02 ^c	1.16±0.04 ^c

Remarks: Specific volumes of bread are expressed as the means ± standard deviations (n = 3). Different letters represent significant differences (p < 0.05)

[00104] Application of BLMA1 on bread baking: Adding 45ppm BLMA1 (6987.6 U/mg) and 60ppm BLMA (3535.0 U/mg) to the bread, the bread formula, baking process and test method were the same as example 15. The hardness and elasticity of the two bread during storage were shown in Figure 7. As the storage days increased, the amount of water loss increased, but the high activity of mutant BLMA1 showed more active effect on starch hydrolysis, and the metabolism of small molecule products increased the water holding capacity of bread. Therefore, the hardness of bread with mutant BLMA1 was lower. In terms of elasticity, the positive effect of mutant BLMA1 was also more significant. After storage of 10 days, the elasticity of bread with BLMA1 increased by about 5%. BLMA1 had more sufficient hydrolysis and produces more effective small molecule products, which reduced the recrystallization rate of starch. In addition, the moisture contained in the bread also makes the bread more elastic. The sensory scores of the bread on the 7th day of storage were shown in Figure 8. The mutant BLMA1 improved the appearance, color, texture, smell and taste of bread, BLMA1 was more conducive to bread moisturizing, the skin color and appearance of the bread would be brighter and more attractive when stored for 7 days, which was more likely to arouse the appetite of the tester for bread. In terms of taste, the better elasticity of bread made the bread taste better. The bread with the 45 ppm BLMA1 maintained better quality during storage, and it would be more economical to apply the mutant BLMA1 to bread baking at a lower concentration.

CLAIMS

What is claimed is:

1. A maltogenic amylase, the amino acid sequence set forth in SEQ ID NO. 1.
2. A gene for coding the maltogenic amylase of claim 1.
3. A recombinant plasmid carrying the gene of claim 2.
4. A host cell carrying the gene of claim 2.
5. A host cell carrying the recombinant plasmid of claim 3.
6. A recombinant microbial cell expressing the maltogenic amylase of claim 1.
7. A recombinant *B. subtilis*, expressing the maltogenic amylase of claim 1, and the recombinant *B. subtilis* takes pMA0911 as an expression vector.
8. A method for soluble expression of maltose amylase, wherein the recombinant *B. subtilis* of claim 7 is inoculated into a medium, and cultured at 30-35°C for 24 to 48 h.
9. The method according to claim 8, wherein the medium contains tryptone, yeast extract, glycerol, potassium dihydrogen phosphate, and dipotassium hydrogen phosphate.
10. An enzyme preparation, wherein it contains the pure maltogenic amylase of claim 1 or a mixture of the maltogenic amylase and an enzyme protective agent.
11. The application of the maltogenic amylase of claim 1, or the recombinant *B. subtilis* of claim 7, or the enzyme preparation of claim 10 in the field of bakery products.

12. The application according to claim 11, wherein the baked products include but is not limited to bread.
13. A maltogenic amylase mutant, comprising an amino acid sequence with mutations of the 418th lysine and the 296th valine, wherein the mutations are relative to a parent amino acid sequence set forth in SEQ ID NO.1.
14. The mutant of claim 13, wherein the amino acid sequence of the maltogenic amylase mutant is set forth in SEQ ID NO.9.
15. A gene for coding the maltogenic amylase mutant of claim 14.
16. A recombinant plasmid carrying the gene of claim 15.
17. A recombinant microbial cell expressing the maltogenic amylase mutant of claim 13.
18. A recombinant *B. subtilis*, expressing the maltogenic amylase mutant of claim 13, and the recombinant *B. subtilis* takes pMA0911 as an expression vector.
19. The application of the mutant of claim 13 or the recombinant *B. subtilis* of claim 18 in the field of bakery products.
20. The application according to claim 19, wherein the bakery products include but is not limited to bread.

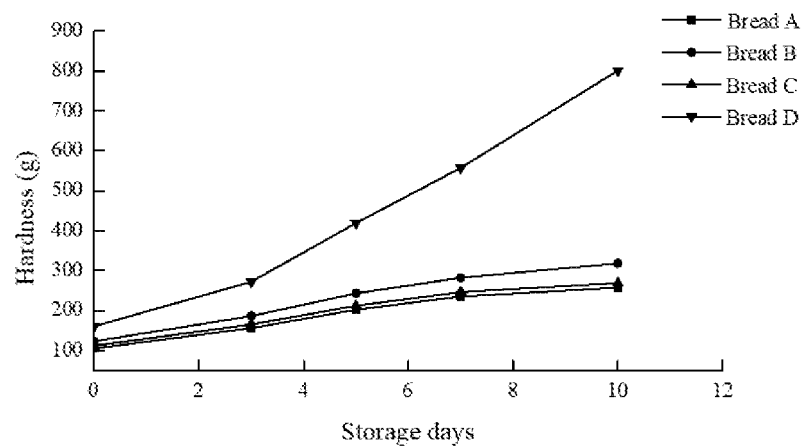


Fig. 1

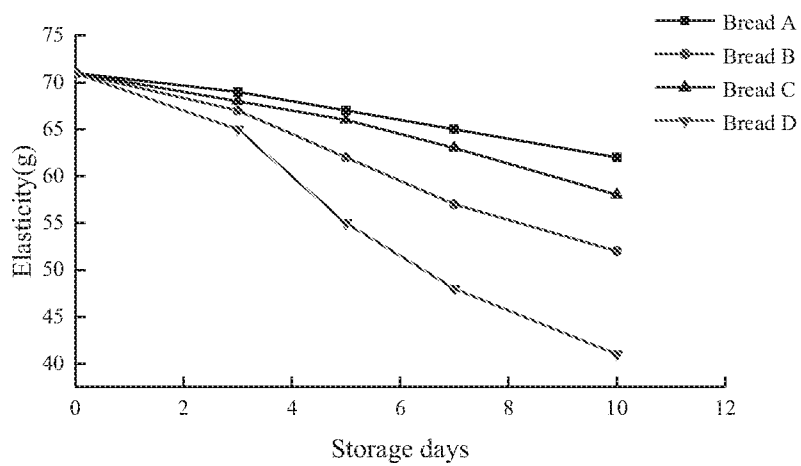


Fig. 2

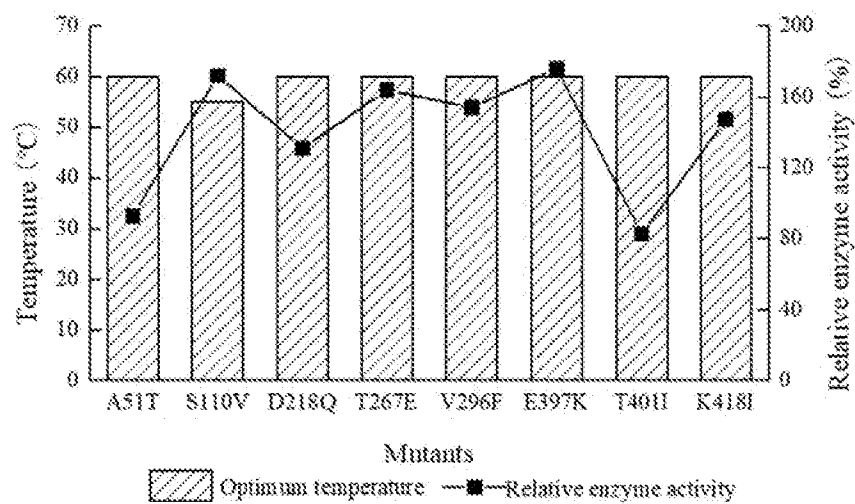


Fig. 3

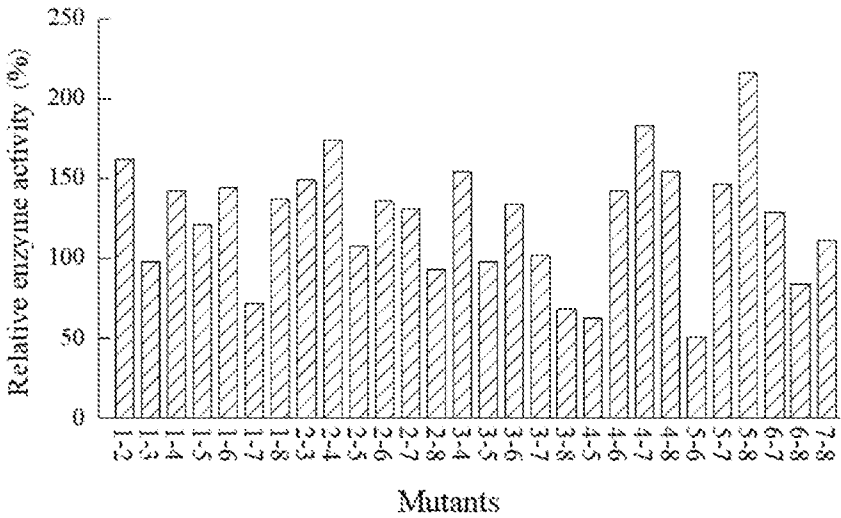


Fig. 4

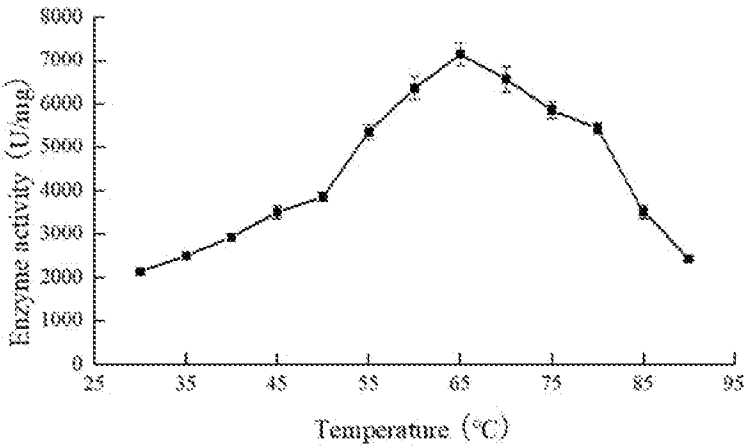


Fig. 5

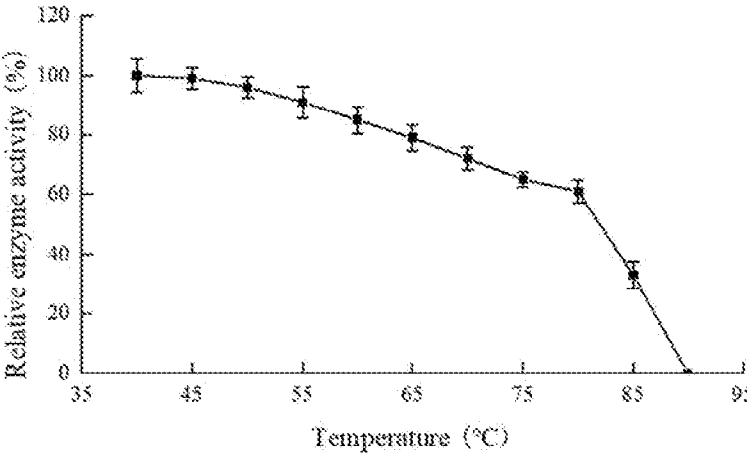


Fig. 6

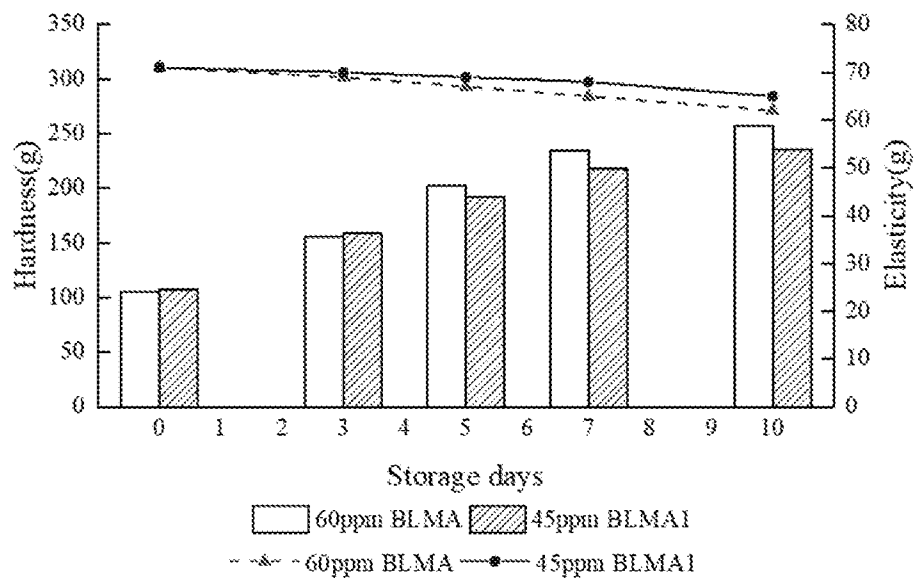


Fig. 7

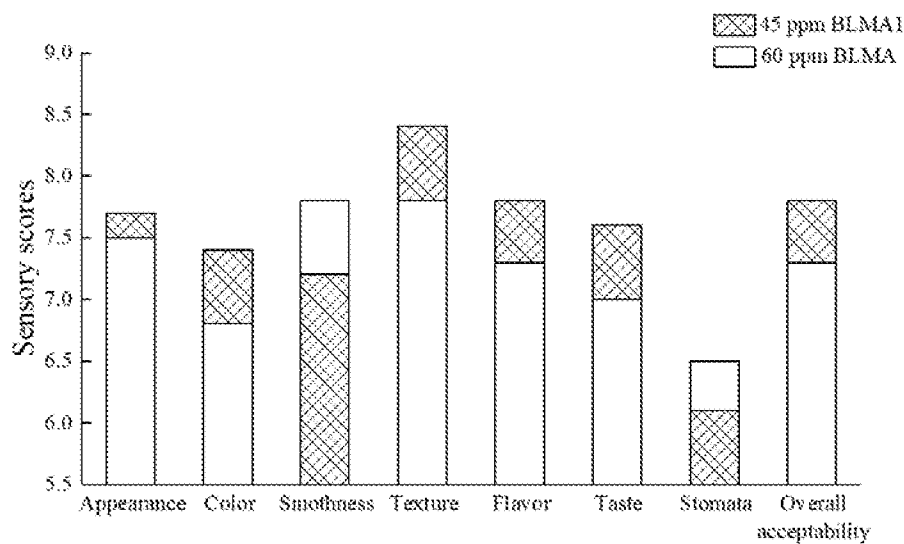


Fig. 8

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2021/103084

A. CLASSIFICATION OF SUBJECT MATTER

C12N 9/28(2006.01)i; A21D 8/04(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N, A21D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DATABASES: DWPI, SIPOABS, CNABS, USTXT, WOTXT, EPTXT, CNTXT, ISI WEB OF KNOWLEDGE, PUBMED, CNKI, DDBJ+EMBL+GENBANK, China Patent Biological Sequence Search System SEARCH ITERM: maltogenic amylase, mutant, bake, bread, 418, 296, SEQ ID NOs:1, 9

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2019193102 A1 (DSM IP ASSETS BV) 10 October 2019 (2019-10-10) see claim 1 and SEQ ID NO:1	1-20
A	CN 111132553 A (NOVOZYMES AS) 08 May 2020 (2020-05-08) see the whole document	1-20
PX	CN 111944785 A (UNIV JIANGNAN) 17 November 2020 (2020-11-17) see claims 1-10	1-20
A	CN 109982576 A (杜邦营养生物科学有限公司) 05 July 2019 (2019-07-05) see the whole document	1-20
A	CN 111148841 A (诺维信公司) 12 May 2020 (2020-05-12) see the whole document	1-20



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

“A” document defining the general state of the art which is not considered to be of particular relevance
 “E” earlier application or patent but published on or after the international filing date
 “L” document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
 “O” document referring to an oral disclosure, use, exhibition or other means
 “P” document published prior to the international filing date but later than the priority date claimed

“T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
 “X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
 “Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
 “&” document member of the same patent family

Date of the actual completion of the international search

01 September 2021

Date of mailing of the international search report

09 October 2021

Name and mailing address of the ISA/CN

National Intellectual Property Administration, PRC
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Facsimile No. (86-10)62019451

Authorized officer

SUN, Yanke

Telephone No. 62411091

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2021/103084

Box No. I **Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2021/103084

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