Overexpression of thermophilic α -amylase in *Bacillus licheniformis* using a high efficiency chromosomal integration and amplification strategy

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Research

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Abstract

Background: The production of industrially important enzymes depends on the development of genetically stable and high-yielding microorganisms. In order to simplify the development of strains able to efficiently overproduce enzymes, a new strategy based on chromosomal integration and amplification in *Bacillus* sp. was developed.

Results: A thermosensitive replicable plasmid pUB-MazF and an integrated expression plasmid pUB'-Ex1 were constructed. pUB-MazF with a thermo-sensitive replicase RepF encoded by gene *repF* can self-replicate in *Bacillus* sp. if the cultivation temperature is no more than 35 °C and if the MazF encoded by *mazF* is not expressed. *mazF* is controlled by an isopropyl β-d-1-thiogalactopyranoside (IPTG)-inducing promoter and MazF is toxic to the cell. When IPTG is present and the MazF encoded by *mazF* in pUB-MazF is expressed, the host cell should lose the plasmid to survive. At a cultivation temperature over 37 °C, pUB-MazF will not well replicate and is not stable. pUB'-Ex1 has a disrupted *repF* and cannot self-replicate but can replicate when pUB-MazF is present. By using this approach, pUB'-Ex1 and constructs can replicate and will be forced to integrate into the host chromosome at 37°C or over when a selectable marker is present. The host was cured by MazF expression after induction with IPTG. *Bacillus licheniformis* BL-UBM integrated with pUB-MazF was transformed with pUB'-amyS derived from pUB'-Ex1 by in-frame cloning of *amyS* encoding a thermophilic α-amylase from *Geobacillus stearothermophilus* ATCC 31195. *B. licheniformis* BL-UBM transformed with pUB'-amyS was cultivated at 42°C with 1 mmol/l IPTG and 500 µg/ml kanamycin and the recombinants expressing high α-amylase activities were selected. All recombinants evaluated were genetically stable. The highest yield of α-amylase of 50 753 U/ml was produced by recombinant BLiS-002 carrying five copies of *amyS* in a 50-l fermenter.

Conclusion: The strategy developed in this study has potential application for convenient and rapid development of strains overexpressing industrially important enzymes.

Introduction

High efficiency preparation of industrially important enzymes depends on development of genetically stable and high-yielding microorganisms, which can present technical difficulties. For the overexpression of a specific enzyme, election or modification of a suitable promoter, signal peptide and terminator, codon optimization and enhancement of the gene copy number in a suitable host cell are the most direct and efficient ways. Two main strategies have been developed to obtain multiple copies of the gene to be expressed. One is based on high copy number expression vectors which replication is under relaxed control. Another is multi-site chromosomal integration or amplification based on *in vivo* DNA homologous recombination. Since the optimized and stable dosage of a gene expressed in the host is a vital factor effecting not only the enzyme expression level but also the physiology and metabolism of the host cells [1, 2], high enzyme expression mediated by high copy number vectors (it is difficult to optimize the gene copies) is seldom achieved for large scale commercial application. Therefore, integrated expression of

the optimal number of gene copies is of great importance in development of enzyme-overproducing strains [3].

Several efficient integrated expression strategies have been developed for enzyme-producing strains over the past decades. Typically, well-developed are site-specific recombination based on homologous recombination and selective chromosomal amplification [4-7]. For example, the *Pichia* expression system is the most well-known system that has been used for many enzyme preparations using a methanol inducing promoter. In that system the *Saccharomyces cerevisiae* alpha-factor signal peptide is mediated secretion where the chromosomal amplification of a target gene is efficiently obtained based on the strength of G418 resistance [8]. In bacterial hosts, 16S rDNA loci have been used as multiple integration sites for certain enzyme-encoding genes; however, this strategy may cause the disruption of 16S rDNAs and subsequently disturb protein synthesis [9].

Alternatively, the methods previously developed for gene editing, deletion or disruption, such as the Cre/*lox* system [10], Xer/*dif* system, FLP/*FRT* system [11] and CRISPR/Cas9 system [6, 12], have also been modified and successfully used for a target gene expression cassette integration at specific loci in the chromosome. These methods can regularly introduce one copy of the gene in every round of genetic manipulation. To prepare strains for industrial enzymes production, many rounds of genetic manipulations are required to obtain multiple copies of the enzyme-encoding gene. In this case, the repeated integration of the same expression cassette is often unsuccessful due to decreased efficiency of site-directed homologous recombination. A CRISPR/cas9 system could be designed with the potential to insert multiple foreign sequences into the host cell chromosomal at the same time [6]. Apart from the same bottlenecks mentioned above, the laboratory work would be challenging to achieve a successful result especially in an industrial host strain as large size recombinant plasmids must first be constructed.

Bacillus licheniformis is an important industrial host with excellent protein synthesis and secretion capacity [13] and many industrial enzymes have been successfully overexpressed and commercially produced in it [2, 14, 15]. However, the genetic instability of integration that is caused by a functional plasmid replication system on the chromosome during the fermentation process is an issue limiting application [16]. To boost the number of specific enzyme-encoding gene copies in a host, a chromosomal amplification strategy based on a pair of replication thermosensitive plasmids was previously developed in *Bacillus lentus* by increasing the protease gene cassette copy number where a thermosensitive replicative plasmid shared the *trans*-acting replication protein with a non-replicative expression plasmid to maintain its replication [16]. The non-replicative expression plasmid could integrate into the chromosome of *B. lentus* after the replicative plasmid was lost when the recombinants grew at elevated cultivation temperature [16]. However, this strategy is troublesome as the replicative plasmid is inefficiently and incompletely lost at least in *B. licheniformis* when temperature is elevated (unpublished data). This may lead to the loss of the integrated expression cassette from the host genome and therefore causes the enzyme overexpression strains to be genetically unstable.

In the present work, an RNase encoded by *mazF* from *Escherichia coli* previously used for scarless gene deletion in *Bacillus* sp. [17] was used as a counter-selection marker to force the complete loss of the replicative plasmid while the integration and amplification of non-replicable expression plasmid in the chromosome occurred. Using this novel strategy, a genetically stable thermophilic α -amylase-overexpressing *B. licheniformis* strain was developed by using only a single generation cycle. To our knowledge, this is the first time that a genetically stable enzyme overexpression *B. licheniformis* strain was efficiently developed. This method may also be used for development of strains that overexpress industrial enzymes with high efficiency.

Results And Discussion

pUB-MazF can assist replication of pUB'-EX1 and can be easily cured

A pair of plasmids, pUB-MazF and pUB'-EX1 (Fig. 1), were constructed as described in method section. pUB-MazF and pUB'-EX1 are *E. coli*-*Bacillus* sp. shuttle vectors and can therefore be used to carry out the genetic manipulation in *E. coli*.

pUB-MazF (Fig. 1a) possesses a functional *repF* gene which encodes a thermosensitive replicase for its automatic replication in *Bacillus* sp. host cells under the incubation temperature no more than 35°C [18]. pUB-MazF also contained a complete *mazF* encoded an endoribonuclease under the control of a lactose-inducing promoter P_{spac} [19]. Since MazF specifically cleaves free mRNAs at the ACA site, its expression leads to the host cell death and therefore only pUB-MazF-cured strain can survive and grow [20].

pUB'-EX1 (Fig. 1b) failed to automatic replicate in the *Bacillus* sp. host cells as the *repF* encoding a replicase was disrupted. Its replication could be restored while pUB-MazF existed and the *repF* functionally expressed. In addition, a 0.5 kb downstream fragment of *B. licheniformis amyL* was inserted in pUB'-EX1 to ensure site-directed integration and amplification in *B. licheniformis* chromosome by homologous recombination under selection pressure.

The replication and stability of pUB-MazF and pUB'-EX1 were checked in both *B. subtilis* and *B. licheniformis* (Fig. 2). pUB-MazF maintained the original thermosensitive replicon of T2(2)-*ori* [21] and replicated independently and stably in both *B. subtilis* and *B. licheniformis* incubated at temperature no more than 35°C. About 20% of populations of cells lost their pUB-MazF when cultivated over 37°C for 72 h in LB medium without tetracycline added (data not shown).

pUB-MazF could be completely cured in the medium supplemented with 1 mmol/l IPTG (Fig. 2). pUB'-EX1 stably replicated in the cell in which pUB-MazF existed and lost or integrated into the chromosome under selection pressure during the cell propagation after pUB-MazF was cured (Fig. 2).

Development of thermophilic α -amylase overexpressing strains based on the cooperation of pUB-MazF and pUB'-EX1

Thermophilic α -amylase overexpressing strains based on the cooperation of pUB-MazF and pUB'-EX1 were developed (Fig. 3) as follows: 1) pUB-MazF was forced to integrate into the chromosome of *B. licheniformis* BL-109 under the selection pressure of 42°C and 2 µg/ml tetracycline yielding *B. licheniformis* recombinant BL-UBM. 2) The gene *amyS* encoding the mature α -amylase of *Geobacillus stearothermophilus* ATCC 31195 was cloned into pUB'-EX1, resulted in pUB'-amyS. It was then transferred into strain BL-UBM and a transformant, renamed as BL-amyS, was selected at 35°C on starch plates containing 2 µg/ml tetracycline and 20 µg/ml kanamycin. 3) Strain BL-amyS was cultivated in LB medium supplemented with 500 µg/ml kanamycin and 1 mmol/l IPTG at 42°C for 48 h. Ten recombinants with different sizes of starch hydrolysis zones were selected and nominated as BLiS001~BLiS010 sequentially. 4) The existence of pUB-MazF was further confirmed by colony PCR using primers P5/P6 and P9/P10 with negative amplification product (data not shown).

The stabilities of recombinants BLiS001~BLiS010 for production of α -amylase were determined. All recombinants maintained α -amylase-producing abilities on starch plates and the same levels of α -amylase activities in shake flask fermentations after 15 generations (Fig. 4a). The results indicated that the recombinants for overexpressing AmyS by the method developed in this study were genetically stable.

It is notable that the recombinants varied in their α -amylase activities, which indicated that the amplification of *amyS* expression cassette in the chromosome occurred. To further confirm the relationship between the copy number of *amyS* and the yield of α -amylase AmyS, the copy number of *amyS* were determined. The results are summarized in Fig. 4a. The recombinants with two copies of *amyS* (recombinants BLiS-001 and BLiS-009) produced about two folds more α -amylase than that of single copy strain BS-109, the recombinants with three copies (recombinants BLiS-003, BLiS-008 and BLiS-010) revealed about three folds more α -amylase. The recombinants with four copies (recombinants BLiS-004, BLiS-006 and BLiS-007) or more (recombinants BLiS-002 and BLiS-005) yielded higher α -amylase activities although the fold increase was slightly reduced (Fig. 4a). These results further illustrated that the number of copies of genes is directly related to the level of enzyme production [2].

The fermentation performance of the recombinant BLiS-002 with the highest yield of the enzyme activity selected in this study was further evaluated in a 50-l fermenter. The results are summarized in Fig. 4b. Strain BLiS-002 produced maximum activity α -amylase of 50 753 U/ml after 72 h cultivation. The expression of α -amylase with a molecular mass of 55.5 kDa was confirmed by SDS-PAGE.

Conclusion

A pair of new plasmids, pUB-MazF and pUB'-EX1, were constructed and a new strategy for gene amplification in *Bacillus* sp. was developed. pUB-MazF works well as an auxiliary plasmid for pUB'-EX1 replication and can be efficiently cured by inducing the expression of MazF after pUB'-EX1 or its constructs were chromosomally integrated. By using the approach, a genetically stable *B. licheniformis* strain for overexpressing a thermophilic α -amylase was quickly developed in only one cycle of genetic manipulation.

Methods

Bacterial strains and plasmids

All the strains and plasmids used in this study are listed in Table 1. *E. coli* JM109 [22] and *Bacillus subtilis* WB600 [23] were used as host cells for DNA manipulation and plasmid DNA preparation. The genome of *B. subtilis* TMO310 integrated *mazF* expression cassette was used as template for amplification of the *mazF* expression cassette [24]. *B. licheniformis* BL-109 derived from *B. licheniformis* CBBD302 [14] by deleting *amyL* encoding a thermophilic alpha-amylase was used as host cell for enzyme overexpression. *B. licheniformis* BS-109 was previously developed in this laboratory by integrating one copy of the thermophilic α -amylase coding sequence *amyS* from *G. stearothermophilus* ATCC 31195 into the *amyL* site of *B. licheniformis* BL-109. All strains were cultivated in LB medium (1% tryptone, 1% NaCl, 0.5% yeast extract) and supplemented with 2 or 5 $\mu\text{g/ml}$ (for *B. licheniformis*) or 8 or 15 $\mu\text{g/ml}$ (for *B. subtilis*) tetracycline or 20-500 $\mu\text{g/ml}$ kanamycin when necessary. LBG medium (LB, 1% glucose) was used to prevent leakage expression of *mazF* by *B. subtilis* and *B. licheniformis* [25], and 1 mmol/l isopropyl-1-thio- β -D-galactopyranoside (IPTG) was applied to induce the expression of *mazF*. The transformants containing the thermophilic α -amylase were selected on starch plates (LB medium containing 1% soluble starch). pT2^{TS} was derived from T2(2)-ori plasmid [21] by reordering the multiple cloning sites from *Bam*HI, *Xba*I and *Sma*I to *Bam*HI, *Sma*I and *Xba*I.

Table 1 Strains and plasmids used in this study

Strains/ Plasmids	Characteristics	Reference
Strains		
<i>E. coli</i> JM 109	<i>endA1, recA1, gyrA96, thi, hsdR17, relA1, supE44, λ⁻, Δ(lac-proAB), [F⁺, traD36, proAB, laqI^qZΔM15]</i>	[22]
<i>B. subtilis</i> WB600	<i>B. subtilis</i> 168, <i>ΔnprE, ΔaprA, Δepr, Δbpf, Δmpr, ΔnprB</i>	[23]
<i>B. subtilis</i> TMO310	<i>aprE::spec, lacI, P_{spac}-mazF</i>	[24]
<i>G. stearothermophilus</i> ATCC 31195	Wild type	Lab stock
<i>B. licheniformis</i> CBBD302	Host cell for gene expression	[14]
<i>B. licheniformis</i> BL-109	<i>B. licheniformis</i> CBBD302, <i>DamyL</i>	Lab stock
<i>B. licheniformis</i> BS-109	<i>B. licheniformis</i> BL-109, <i>amyL::amyS</i>	Lab stock
<i>B. licheniformis</i> UBM	Chromosome-integrated pUB-MazF in <i>B. licheniformis</i> BL-109	This study
<i>B. licheniformis</i> amyS	<i>B. licheniformis</i> BL-UBM, pUB'-amyS	This study
<i>B. licheniformis</i> BLiS001	<i>B. licheniformis</i> BL-109, 2 copies of <i>amyS</i>	This study
<i>B. licheniformis</i> BLiS002	<i>B. licheniformis</i> BL-109, 5 copies of <i>amyS</i>	This study
<i>B. licheniformis</i> BLiS003	<i>B. licheniformis</i> BL-109, 3 copies of <i>amyS</i>	This study
<i>B. licheniformis</i> BLiS004	<i>B. licheniformis</i> BL-109, 4 copies of <i>amyS</i>	This study
<i>B.</i>	<i>B. licheniformis</i> BL-109, 5 copies of <i>amyS</i>	This

<i>licheniformis</i> BLiS005		study
<i>B. licheniformis</i> BLiS006	<i>B. licheniformis</i> BL-109, 4 copies of <i>amyS</i>	This study
<i>B. licheniformis</i> BLiS007	<i>B. licheniformis</i> BL-109, 4 copies of <i>amyS</i>	This study
<i>B. licheniformis</i> BLiS008	<i>B. licheniformis</i> BL-109, 3 copies of <i>amyS</i>	This study
<i>B. licheniformis</i> BLiS009	<i>B. licheniformis</i> BL-109, 2 copies of <i>amyS</i>	This study
<i>B. licheniformis</i> BLiS010	<i>B. licheniformis</i> BL-109, 3 copies of <i>amyS</i>	This study
plasmids		
pT2 ^{TS}	Km ^R , derived from T2(2)-ori, <i>E. coli-Bacillus</i> sp. shuttle vector, thermosensitive replication	This study
pHY-WZX	Tet ^R , Ap ^R and Km ^R , expression vector	[14]
pHY300PLK	Tet ^R , Ap ^R , <i>E. coli-Bacillus</i> sp. shuttle plasmid	[26]
pUB-EX	Km ^R , derived from pT2 ^{TS} , harboring the expression cassette of pHY-WZX,	This study
pUB-Tet	Tet ^R , derived from pUB-EX	This study
pUB-MazF	Tet ^R , replication thermosensitive <i>E. coli-Bacillus</i> sp. shuttle vector, carrying P _{spac} controlled the transcription of <i>mazF</i>	This study
pUB'-sint	Km ^R , $\Delta repF$, derived from pUB-EX,	This study
pUB'-Ex1	Km ^R , thermosensitive and non-replicable independently plasmid in <i>Bacillus</i> sp.	This study
pUB'-amyS	Km ^R , pUB'-Ex1 carrying α -amylase expression cassette <i>amyS</i> from <i>G. stearothermophilus</i> ATCC 31195	This study

Construction of pUB-MazF and pUB'-EX1

The expression cassette from plasmid pHY-WZX [15] containing the promoter and signal peptide-encoding the *B. licheniformis amyL* sequence, multiple cloning site and artificial terminator was isolated by PCR amplification with primers P1 and P2 (Table S1). The PCR product was digested by *Bam*HI and *Avr*II then cloned into *Bam*HI and *Xba*I sites of pT2^{TS} to yield a recombinant plasmid pUB-EX. It was then used to construct pUB-MazF and pUB'-EX1. For the construction of pUB-MazF, the kanamycin resistance cassette was removed by reverse PCR using primers P3 and P4. The resulting fragment was ligated with tetracycline resistance fragment isolated from pHY300PLK by using primers P5 and P6 to yield recombinant plasmid pUB-Tet. The *mazF* cassette was recovered from the chromosome of *B. subtilis* TMO310 by PCR amplification using primers P9 and P10 and then cloned into the *Sma*I site of pUB-Tet to yield recombinant plasmid pUB-MazF. For construction of pUB'-EX1, the *repF* encoding a replicase in pUB-EX was disrupted by reverse PCR using primers P7 and P8 and the product was self-ligated to yield an intermediate plasmid pUB'-sint. A 0.5 kb downstream fragment of *amyL* was amplified from *B. licheniformis* CBBD302 chromosome using primers P11 and P12 and cloned into *Bam*HI site of pUB'-sint, resulted in integrated expression plasmid pUB'-EX1. The primers used in this study are listed in Supplementary Table S1.

Examination of replication properties of pUB-MazF and pUB'-EX1

pUB-MazF and pUB'-EX1 were transformed into *B. subtilis* WB600 using the method described by Anagnostopoulos & Spizizen [27]. The transformants carrying pUB-MazF or both pUB-MazF and pUB'-EX1 were grown in LB with or without 1 mmol/l IPTG at 35°C or 42°C. The cultures were then diluted and spread on the LB plates supplemented with 8 µg/ml tetracycline and/or 20 µg/ml kanamycin. After incubation for 24~48 h at 35°C, the growth properties were recorded. pUB-MazF and pUB'-EX1 were transformed into *B. licheniformis* CBBD302 by electro-transformation as described by Xu et al [28]. The replication properties of pUB-MazF and pUB'-EX1 in *B. licheniformis* CBBD302 were recorded as described above.

Overexpression of *G. stearothermophilus* alpha-amylase in *B. licheniformis*

The *amyS* encoding the mature peptide of a thermophilic α-amylase was recovered from the genome of *G. stearothermophilus* ATCC 31195 by PCR amplification with primers P13 and P14. The PCR product was digested by *Xba*I and cloned into *Xba*I and *Sma*I sites of pUB'-EX1, yielding a recombinant plasmid pUB'-amyS. pUB-MazF was electro-transformed into BL-109 and randomly integrated into the chromosome by cultivating the transformants at 42°C on LB supplemented 2 µg/ml tetracycline, yielding recombinant strain BL-UBM. It was then transformed with pUB'-amyS to obtain the transformant BL-amyS (*B. licheniformis* BL-UBM, pUB'-amyS). The strain BL-amyS was then cultivated at 42°C in LB supplemented with 500 µg/ml kanamycin and 1 mmol/l IPTG. The culture was diluted and spread on

starch plates and the recombinants with large starch-hydrolyzing zone but no growth on LBG plates with 2 µg/ml tetracycline were selected and were kept for evaluation of genetic stability and α-amylase yields.

Measurement of the *amyS* copy number in recombinants

The *amyS* copy number in recombinants chromosome was quantified by the real-time qPCR method with SYBR Green dye as a marker. Primer pair P15/P16 was used for amplification and the measurement was carried out in triplicate in an ABI StepOnePlus Real-Time PCR system. The copy number was calculated by $2^{-\Delta C_t}$ method as described previously [2] with *B. licheniformis* BS-109 with one copy of *amyS* as control.

Assessment of recombinant stability

The stability of recombinants was measured as follows: 1 % of the culture was transferred to fresh liquid LB medium, grown for 12 hours when sample of the culture was taken and spread onto starch plates or used to inoculate fresh LB medium. This process was repeated fifteen times. The size of halos and percentage of strains with haloes on starch plates were calculated. The cultures of each generation were assayed the enzyme activity as described below.

Fermentation experiment

The flask fermentation was carried out as described as previous study [2] with slight modification. Briefly, a single colony of a recombinant strain was inoculated into LB medium and cultured over-night at 200 rpm and 37°C until the late log phase. Five ml of the culture was inoculated into a 250 ml flask containing 50 ml fermentation medium. The fermentation was carried out at 37°C and 220 rpm for 120 h.

A scaled-up fermentation was carried out in a 50-l fermenter to further evaluate enzyme expression level of the selected recombinant. The fermentation conditions were described previously [14]. Culture samples were taken every 4 h and the supernatant was isolated by centrifugation. The enzyme activity was measured.

Enzyme activity assay and other analytic methods

The activity of the thermophilic α-amylase was determined as described by Hollo and Szeitli [29]. One unit of the enzyme is defined as the amount of enzyme needed to hydrolyze 1 mg soluble starch per minute at 70°C and pH 6.0. The optical density was measured at 600 nm in triplicate with an SP-2012UV spectrophotometer (Shanghai Spectrum Instruments, China). The amount of enzyme protein was determined by SDS-PAGE method stained by Coomassie Brilliant Blue G250 [30].

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

DN and ZW conceived the study. PS, DN, PK, XL and KT conducted the experiments. PS, DN, PK, SS, and ZW analyzed the data and prepared the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

On behalf of all authors, the corresponding authors state that there is no conflict of interest.

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Figures

Figure 1

The physical maps of pUB-MazF (a) and pUB'-EX1 (b). pUB-MazF and pUB'-EX1 are *E. coli*-*Bacillus* sp. shuttle vectors. pUB-MazF had the functional *repF* which encodes a thermosensitive replicase for its automatic replication in *Bacillus* sp. host cells under the incubation temperature no more than 35°C. pUB-MazF also contained a complete *mazF* encoded an endoribonuclease under the control of a lactose-inducing promoter *Pspac*. The *repF* in pUB'-EX1 had been disrupted and its replication could only be restored while pUB-MazF existed and its *repF* functionally expressed. A 0.5 kb downstream fragment of *B. licheniformis* *amyL* (3'-*amyL'*) was inserted in pUB'-EX1 to make it site-directed integration and amplification in *B. licheniformis* chromosome.

Figure 2

The confirmation of the replicon properties of pUB-MazF and pUB'-EX1 in *B. subtilis* and *B. licheniformis*. All strains were cultivated in LB with no antibiotic added for 48 h, the cultures were diluted and spread on LB plate complemented kanamycin and/or tetracycline and cultivated for 48 h. a: pUB-MazF in *B. subtilis* WB600 or *B. licheniformis* BL-109. b: The property of pUB'-EX1 was confirmed in *B. subtilis* WB600 or *B. licheniformis* BL-109 harboring free pUB-MazF. c: IPTG was added in LB medium when cultured recombinant *B. subtilis* and *B. licheniformis* and the concentration was 1 mmol/l.

Figure 3

The flowchart of α -amylase-overexpressing strain development based on pUB-MazF and pUB'-EX1. pUB-MazF was transformed into *B. licheniformis* BL-109 and then forced to integrate into the chromosome under the selection pressure of 42oC and 2 µg/ml tetracycline, yielded a *B. licheniformis* recombinant BL-UBM. pUB'-amyS derived from pUB'-EX1 by cloning *amyS* encoding the mature thermophilic α -amylase from *G. stearothermophilus* ATCC 31195 was transferred into strain BL-UBM at 35oC on starch plates containing 2 µg/ml tetracycline and 20 µg/ml kanamycin and obtained transformant BL-amyS. Recombinants BLiS-001~BLiS-010 were selected by incubating strain BL-amyS in LB medium

supplemented 500 µg/ml kanamycin and 1 mmol/l IPTG at 42°C for 48 h and subsequently spread onto starch plates for another 24 h.

Figure 4

Genetic stability, copy number of amyS and fermentation performance of the recombinant *B. licheniformis*. a: The yields of α -amylase after 15 generations of ten recombinant strains with different copy numbers of amyS, open column: first generation, solid column: 15th generation; b: The time-course of AmyS production by recombinant *B. licheniformis* strain BLiS-002 during fermentation in a 50-l fermenter, embedded box: SDS-PAGE profile of AmyS taken from the fermentation broth at 72 h.

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