Journal of Industrial Microbiology and Biotechnology, 2022, 49, kuac009

https://doi.org/10.1093/jimb/kuac009
Advance access publication date: 23 March 2022
Genetics and Molecular Biology of Industrial Organisms – Original Paper

High-efficiency chromosomal integrative amplification strategy for overexpressing α-amylase in *Bacillus licheniformis*

Peili Shen[1], Dandan Niu[2], Xuelian Liu[2], Kangming Tian[2], Kugen Permaul[3], Suren Singh[3], Nokuthula Peace Mchunu[4], Zhengxiang Wang[1,2]

[1]College of Biotechnology, Tianjin University of Science and Technology, Tianjin 300457, China
[2]Department of Biological Chemical Engineering, College of Chemical Engineering and Materials Science, Tianjin University of Science and Technology, Tianjin 300457, China
[3]Department of Biotechnology and Food Science, Faculty of Applied Sciences, Durban University of Technology, PO Box 1334, Durban 4001, South Africa
[4]Biotechnology Platform, Agricultural Research Council, Private Bag X5, Onderstepoort 0110, South Africa
Correspondence should be addressed to: Zhengxiang Wang, E-mail: zxwang0519@tust.edu.cn; Dandan Niu, E-mail: ddniu@tust.edu.cn

Abstract: *Bacillus licheniformis* is a well-known platform strain for production of industrial enzymes. However, the development of genetically stable recombinant *B. licheniformis* for high-yield enzyme production is still laborious. Here, a pair of plasmids, pUB-MazF and pUB'-EX1, were firstly constructed. pUB-MazF is a thermosensitive, self-replicable plasmid. It was able to efficiently cure from the host cell through induced expression of an endoribonuclease MazF, which is lethal to the host cell. pUB′-EX1 is a nonreplicative and integrative plasmid. Its replication was dependent on the thermosensitive replicase produced by pUB-MazF. Transformation of pUB′-EX1 into the *B. licheniformis* BL-UBM harboring pUB-MazF resulted in both plasmids coexisting in the host cell. At an elevated temperature, and in the presence of isopropyl-1-thio-β-d-galactopyranoside and kanamycin, curing of the pUB-MazF and multiple-copy integration of pUB′-EX1 occurred, simultaneously. Through this procedure, genetically stable recombinants integrated multiple copies of *amyS*, from *Geobacillus stearothermophilus* ATCC 31195 were facilely obtained. The genetic stability of the recombinants was verified by repeated subculturing and shaking flask fermentations. The production of α-amylase by recombinant BLiS-002, harboring five copies of *amyS*, in a 50-l bioreactor reached 50 753 U/ml after 72 hr fermentation. This strategy therefore has potential for production of other enzymes in *B. licheniformis* and for genetic modification of other *Bacillus* species.

Keywords: Chromosomal integration, MazF, *Bacillus licheniformis*, Overexpression, α-Amylase

Introduction

Efficient production of industrially important enzymes depends on the development of genetically stable and high-yielding host microbial strains. To meet this industrial requirement, recombinant DNA technology has been widely used for modification of enzyme production strains. For the overexpression of a specific industrial enzyme in a suitable host cell, the most direct and efficient ways are as follows: selection or modification of a suitable promoter (Öztürk et al., 2017), selection of a suitable and optimized signal peptide for secretion (Wang et al., 2014), codon optimization (Al-Hawash et al., 2017), and enhancement of the gene copy number (Nadler et al., 2019). Two main strategies have been developed to express multiple copies of the target gene. One strategy is based on high-copy-number expression vectors. The other is multisite chromosomal integration based on DNA homologous recombination *in vivo*. The proper and stable gene dosage in the host cell is a vital factor, which affects not only the enzyme expression level but also the physiology and metabolism of the host cell (Niu et al., 2009). High enzyme expression using high-copy-number vectors is seldom used for large-scale commercial applications (it is unstable and difficult to optimize the gene copies). Therefore, integrated expression of the optimal number of gene copies is of great importance for the development of enzyme-overproducing strains.

Previously, several efficient integrated expression strategies have been developed for enzyme-producing strains. One of the well-developed strategies is site-specific recombination based on homologous recombination (Dong & Zhang, 2014; Li et al., 2018; Macauley-Patrick et al., 2005; Zakataeva et al., 2010). For example, the *Pichia* expression system is the most well-known system that has been used for enzyme preparation using a methanol-inducible promoter. In that system, the chromosomal amplification of a target gene is efficiently obtained based on the strength of G418 resistance, and the *Saccharomyces cerevisiae* α-factor signal peptide is used to mediate secretion of the target protein (Scorer et al., 1994). In *Bacillus subtilis*, the 16S rDNA locus was used as multiple integration sites for certain enzyme-encoding genes. However, this strategy may cause the disruption of 16S rDNA and subsequently disturb protein synthesis (Yano et al., 2013).

Alternative previously developed methods for *Bacillus* sp. gene editing, deletion, or disruption, for instance the Cre/*loxP* system (Yan et al., 2008), Xer/*dif* system (Bloor & Cranenburgh, 2006), FLP/*FRT* system (Li et al., 2019), and CRISPR/Cas9 system (Li et al., 2018; Zhou et al., 2019), have also been modified and successfully used for target gene expression cassette integration into specific

Received: January 12, 2022. **Accepted:** March 14, 2022.

Table 1. Strains and Plasmids Used in This Study

Strains/Plasmids	Characteristics	Resource
Strains		
E. coli JM109	*endA1*, *recA1*, *gyrA96*, *thi*, *hsdR17*, *rel*A1, *sup*E44, λ$^-$, Δ(*lac-pro*AB), [F′, *tra*D36, *pro*AB, *laqI*^{q}ZΔM15]	Lab stock
B. subtilis WB600	*B. subtilis* 168, Δ*nprE*, Δ*aprA*, Δ*epr*, Δ*bpf*, Δ*mpr*, Δ*nprB*	Lab stock
B. subtilis TMO310	*aprE*::*spec*, *lacI*, P_{spac}-*mazF*	Morimoto et al. (2009)
G. stearothermophilus ATCC 31195	Wild type	Lab stock
B. licheniformis CBBD302	Host cell for gene expression	Niu et al., (2009b)
B. licheniformis BL-109	*B. licheniformis* CBBD302, Δ*amyL*	Lab stock
B. licheniformis BS-109	*B. licheniformis* BL-109, *amyL*::*amyS*	Lab stock
B. licheniformis BL-UBM	Chromosome-integrated pUB-MazF in *B. licheniformis* BL-109	This study
B. licheniformis BL-amyS	*B. licheniformis* BL-UBM, pUB′-amyS	This study
B. licheniformis BLiS-001	*B. licheniformis* BL-109, two copies of *amyS*	This study
B. licheniformis BLiS-009	*B. licheniformis* BL-109, two copies of *amyS*	This study
B. licheniformis BLiS-003	*B. licheniformis* BL-109, 3 copies of *amyS*	This study
B. licheniformis BLiS-008	*B. licheniformis* BL-109, three copies of *amyS*	This study
B. licheniformis BLiS-010	*B. licheniformis* BL-109, three copies of *amyS*	This study
B. licheniformis BLiS-004	*B. licheniformis* BL-109, four copies of *amyS*	This study
B. licheniformis BLiS-006	*B. licheniformis* BL-109, four copies of *amyS*	This study
B. licheniformis BLiS-007	*B. licheniformis* BL-109, four copies of *amyS*	This study
B. licheniformis BLiS-002	*B. licheniformis* BL-109, five copies of *amyS*	This study
B. licheniformis BLiS-005	*B. licheniformis* BL-109, five copies of *amyS*	This study
Plasmids		
pHY300PLK	Tet^R, Ap^R, *E. coli–Bacillus* sp. shuttle plasmid	Ishiwa & Shibahara, (1985)
pHY-WZX	Tet^R, Ap^R, Km^R, expression vector	Niu & Wang (2007)
pUB-EX	Km^R, thermosensitive plasmid, harboring the expression cassette of pHY-WZX	Lab stock
pUB-Tet	Tet^R, derived from pUB-EX	This study
pUB-MazF	Tet^R, replication-thermosensitive *E. coli–Bacillus* sp. shuttle vector, carrying P_{spac} controlled the transcription of *mazF*	This study
pUB′-sint	Km^R, Δ*repF*, derived from pUB-EX	This study
pUB′-EX1	Km^R, integrative vector, thermosensitive and nonreplicable independent plasmid in *Bacillus* sp.	This study
pUB′-amyS	Km^R, integrated expression plasmid, pUB′-EX1 carrying α-amylase expression cassette *amyS* from *G. stearothermophilus* ATCC 31195	This study

loci in the chromosome. To construct recombinant strains carrying multiple copies of a target gene, multiple genetic operations using the aforementioned gene integration methods are required. The operation process is complex and time-consuming.

Bacillus licheniformis is an important industrial host with excellent protein synthesis and secretion capacity (Schallmey et al., 2004) and many industrial enzymes have been successfully overexpressed and commercially produced in this host cell (Niu et al., 2009; Niu & Wang, 2007). However, the genetic instability that is caused by functional plasmid replication systems in the chromosome during the fermentation process is an issue limiting application (Young & Ehrlich, 1989). In a previous study, a chromosomal amplification strategy based on a pair of thermosensitive plasmids was developed to boost the number of specific enzyme-encoding gene copies in *Bacillus lentus* (Jørgensen et al., 2000). In that method, a replication-thermosensitive plasmid, pE194, acted as a helper plasmid to restore the temporary replication of a nonreplicative expression plasmid pPL2002, which leads to higher integration efficiency of plasmid pPL2002 or its derivatives in the *B. lentus* chromosome. The helper plasmid, pE194, was then cured by raising the cultivation temperature (Jørgensen et al., 2000). However, this strategy is not suitable for *B. licheniformis* due to being inefficient and incomplete for plasmid curing during our research process.

In the present work, an RNase encoded by *mazF* from *Escherichia coli* was used as a counterselection marker and its expression was lethal to the host cell (Zhan et al., 2003). It was used to force the complete loss of the replicative plasmid, while the integration and amplification of the nonreplicable expression plasmid occurred in the chromosome. Using this novel strategy, a genetically stable, thermophilic α-amylase-overexpressing *B. licheniformis* strain was developed using only a single generation cycle. To our knowledge, this is the first time that a high-efficiency chromosomal integration strategy has been developed in a *B. licheniformis* strain. This method may also be used for the development of strains to overexpress other industrial enzymes with high yields.

Materials and Methods

Bacterial Strains and Plasmids

The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* JM109 was used as the host cell for DNA manipulation and plasmid DNA preparation. *Bacillus subtilis* WB600 was used as one of the host cells to investigate the properties

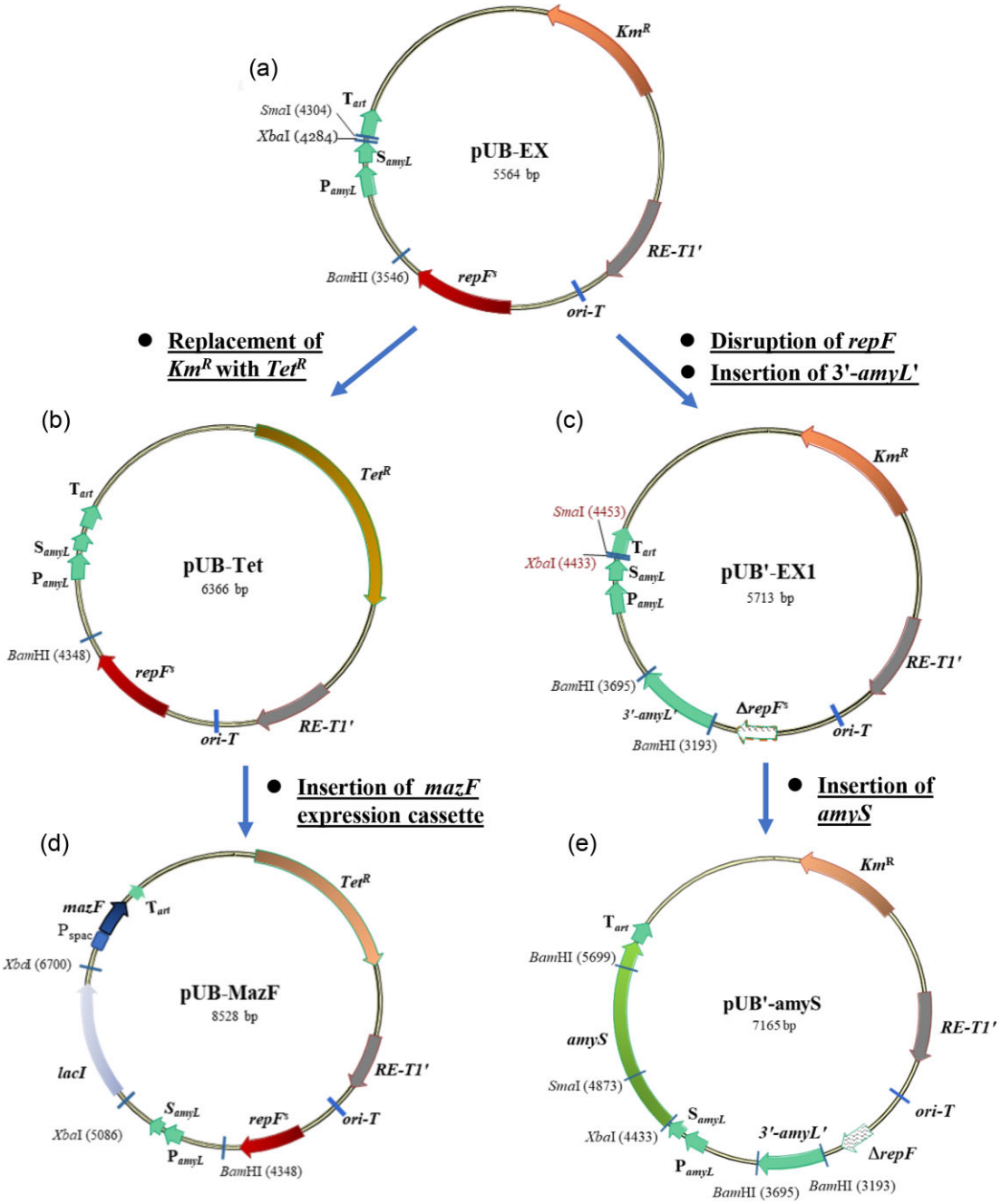

Fig. 1 The flowchart for construction of new plasmids. The kanamycin resistance cassette of parent plasmid pUB-EX (a) was removed by inverse PCR using primers P1 and P2. The resulting fragment was ligated with a tetracycline resistance cassette fragment isolated from pHY300PLK by PCR using primers P3 and P4 to yield helper plasmid pUB-Tet (b). The *repF* encoding a replicase in pUB-EX was disrupted by inverse PCR using primers P5 and P6 and the product was self-ligated to yield an intermediate plasmid pUB′-sint. A downstream fragment of *amyL*, 3′-*amyL*′, was amplified from *B. licheniformis* CBBD302 chromosome using primers P9 and P10. It was inserted into the *Bam*HI site of pUB′-sint as a homologous arm to yield integrative expression plasmid pUB′-EX1 (c). The *mazF* cassette was recovered from the chromosome of *B. subtilis* TMO310 by PCR amplification using primers P7 and P8 and cloned into the *Sma*I site of pUB-Tet to obtain helper plasmid pUB-MazF (d). The *amyS* encoding the thermophilic α-amylase from *G. stearothermophilus* ATCC 31195 was isolated by PCR amplification with primers P11 and P12, and the PCR product was cloned into the *Xba*I and *Sma*I sites of pUB′-EX1 after being digested by *Xba*I, yielding α-amylase integrative expression plasmid pUB′-amyS (e). The elements in the vectors as follows: *repF*, encoding a thermosensitive replicase, Δ*repF*, represents the nonfunctional thermosensitive replicase gene sequence; P_{amyL}, S_{amyL}, and T_{art} represent the promoter, signal peptide, and terminator form pHY-WZX; and *ori-T* represents the temperature-sensitive replication origin.

of the plasmids. The genome of *B. subtilis* TMO310 was used as the template for amplification of the isopropyl-1-thio-β-D-galactopyranoside (IPTG)-induced *mazF* expression cassette, in which the *E. coli mazF* was cloned after the IPTG-inducible promoter P_{spac} and integrated in the chromosome of *B. subtilis* TMO310 (Morimoto et al., 2009). *Bacillus licheniformis* BL-109 derived from *B. licheniformis* CBBD302 (Niu et al., 2009) by deleting the thermophilic α-amylase coding gene *amyL* was used as the host cell for enzyme overexpression. *Bacillus licheniformis* BS-109 was previously developed in this laboratory by integrating one copy of *amyS*

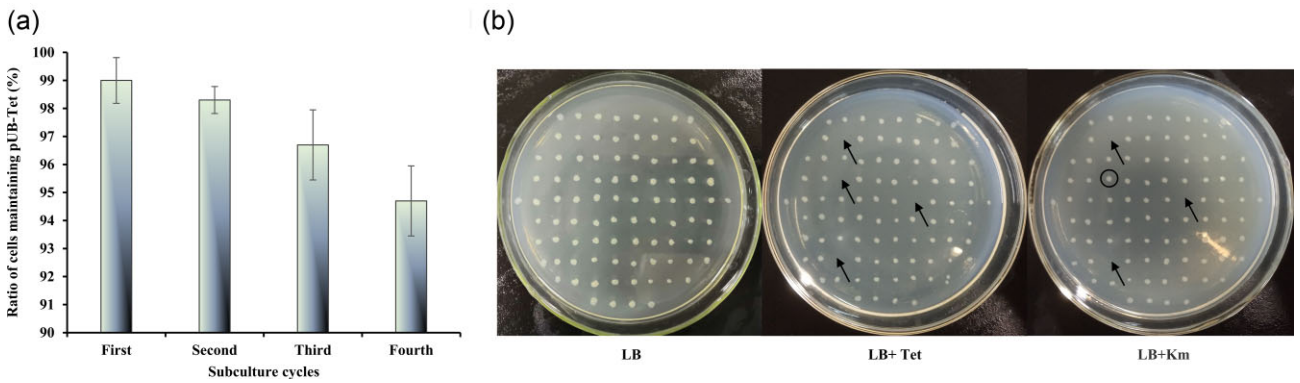

Fig. 2 The helper plasmid pUB-Tet curing and the expression plasmid pUB′-EX1 integration in *B. licheniformis*. (a) The ratio of cells maintaining helper plasmid after 4 times subculturing; error bars indicate standard deviation from three parallel experiments. (b) The evaluation of plasmid pUB-Tet and pUB′-EX1 existence in *B. licheniformis*. The concentration of tetracycline in LB plates was 2 μg/ml and the concentration of kanamycin in the LB plate was 20 μg/ml. Arrows indicate colonies with helper plasmid curing; the open circle marks the colony in which pUB′-EX1 was integrated into the chromosome.

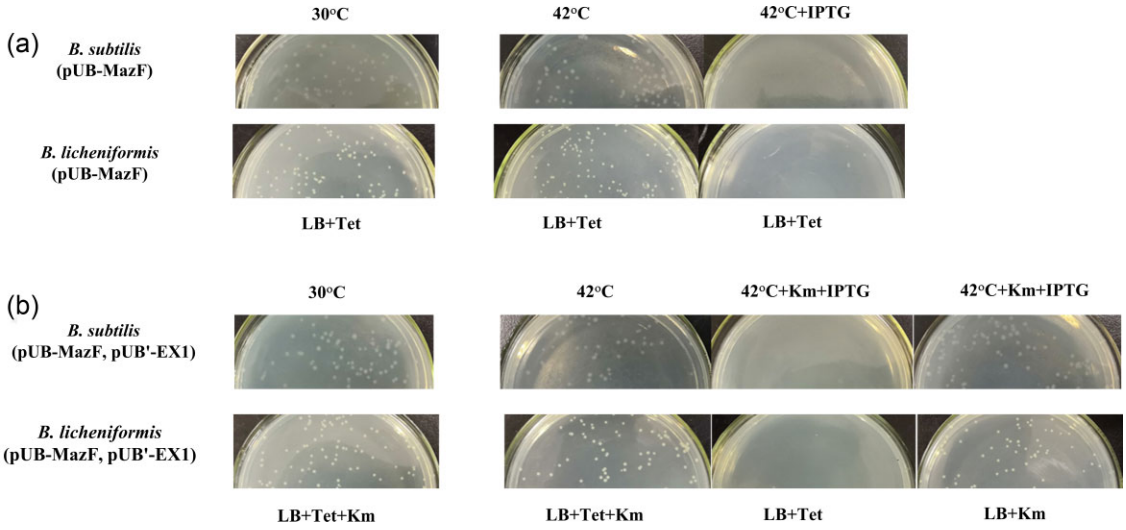

Fig. 3 Functional verification of pUB-MazF and pUB′-EX1 in *B. subtilis* and *B. licheniformis*. (a) The replication of pUB-MazF in *B. subtilis* WB600 and *B. licheniformis* BL-109. (b) The replication and integration of pUB′-EX1 with the assistance of pUB-MazF in *B. subtilis* WB600 and *B. licheniformis* BL-109. Culture conditions were marked at the top of each section. For verification of plasmid replication and integration, the cultures were spread on LB plates containing tetracycline (15 μg/ml in *B. subtilis* or 5 μg/ml in *B. licheniformis*) and/or 20 μg/ml kanamycin.

encoding the mature peptide of a thermophilic α-amylase from *Geobacillus stearothermophilus* ATCC 31195. The strains were cultivated at 37°C in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) and supplemented with 2 or 5 μg/ml (for *B. licheniformis*) and 8 or 15 μg/ml (for *B. subtilis*) tetracycline or 20 μg/ml kanamycin when necessary. The expression of MazF was controlled by P_{spac} and could be induced with 1 mmol/l IPTG. pUB-EX was a thermosensitive expression vector stored in our lab. pUB-MazF and pUB′-EX1 were constructed from pUB-EX in this study.

DNA Manipulation

Conventional DNA manipulations, chromosomal DNA isolation, polymerase chain reaction (PCR), plasmid DNA extraction, restriction endonuclease digestion, ligation, and transformation of *E. coli* were performed according to the convenient protocols (Sambrook & Russel, 2001). The primers used in this study are listed in Table S1.

Genetic Transformation

Genetic transformation of *B. subtilis* WB600 was carried out using the method described by Anagnostopoulos & Spizizen (1961). Genetic transformation of *B. licheniformis* CBBD302 was done by electrotransformation described by Xu et al. (2004). The transformants were screened on LB plates supplemented with 15 μg/ml (for *B. subtilis*) or 2 μg/ml (for *B. licheniformis*) tetracycline with or without 20 μg/ml kanamycin at 30°C. When necessary, the transformants were further screened at 42°C.

Verification of pUB-MazF and pUB′-EX1 Replication Ability

The transformants of *B. subtilis* WB600 carrying pUB-MazF or both pUB-MazF and pUB′-EX1 were subcultivated in LB medium with or without 1 mmol/l IPTG at 30 and 42°C with shaking at 200 rpm for 48 hr. Every 12 hr, 2% of the inoculum was transferred to fresh LB medium. The cultures were then diluted and spread on the LB

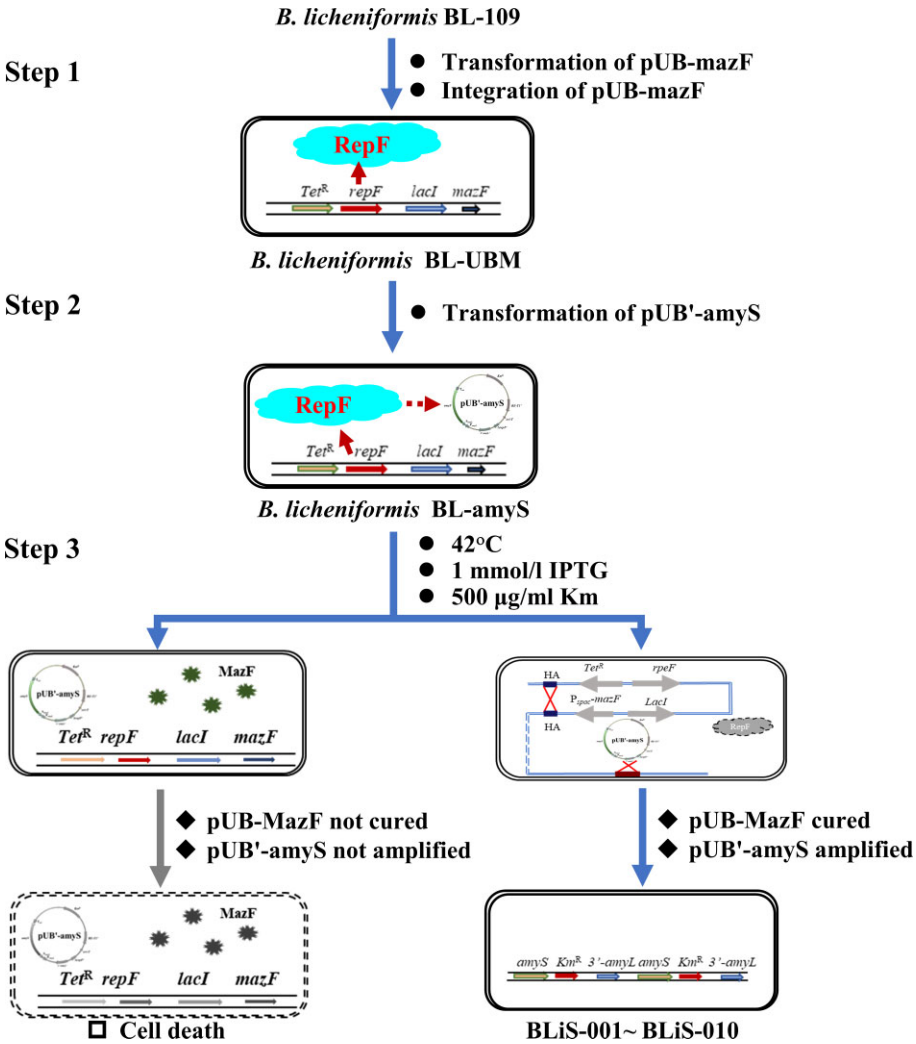

Fig. 4 The flowchart of α-amylase-overexpressing strain development. *Bacillus licheniformis* BL-UBM integrated with pUB-MazF (step 1) was transformed with pUB′-amyS (step 2) to obtain transformant BL-amyS. The α-amylase-overexpressing strains (step 3) with multiple copies of *amyS* in the chromosome were screened when supplemented with 1 mmol/l IPTG and 500 μg/ml Km.

plates supplemented with 15 μg/ml tetracycline and/or 20 μg/ml kanamycin at 30°C for 48 hr; the replication ability of pUB-MazF and pUB′-EX1 in *B. subtilis* WB600 was determined by recording the growth properties of the transformants.

The replication ability of pUB-MazF and pUB′-EX1 in *B. licheniformis* BL-109 was recorded as described earlier. The tetracycline concentration in medium changed to 5 μg/ml for *B. licheniformis* CBBD302 using in this study is sensitive to tetracycline (Niu et al., 2009b).

Development of Recombinants Expressing *G. stearothermophilus* α-Amylase

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 P11 and P12. The PCR product was digested by *XbaI* and cloned into the *XbaI* and *SmaI* sites of pUB′-EX1, yielding the recombinant plasmid pUB′-amyS (Fig. 1e), used to deliver the *amyS* into the host cell.

The resulting pUB′-amyS was transformed into *B. licheniformis* BL-UBM harboring pUB-MazF. The transformant *B. licheniformis* BL-UBM (pUB′-amyS) was obtained at 30°C in starch plates (LB plates containing 1% starch) supplemented with 20 μg/ml kanamycin and 2 μg/ml tetracycline and then was further cultivated at 42°C and 200 rpm in LB medium supplemented with 500 μg/ml kanamycin and 1 mmol/l IPTG. The target integrated transformants were screened by spotting colonies on LB plates supplemented with 2 μg/ml tetracycline and starch plates containing 500 μg/ml kanamycin, respectively. The colonies that only grew on starch plates were selected and further evaluated by colony PCR using primer pair P9/P10.

Measurement of the *amyS* Copy Number in Recombinants

The copy number of *amyS* in the recombinants was quantified by the qPCR method with SYBR Green dye as the fluorescent label; the primer pair P13/P14 was used for the amplification reaction and the measurement was carried out in triplicate in an ABI StepOnePlus™ Real-Time PCR system (Applied Biosystems®). The copy number was calculated by the $2^{-\Delta Ct}$ method as described previously (Niu et al, 2009), using *B. licheniformis* BS-109 with one copy of *amyS* as the control.

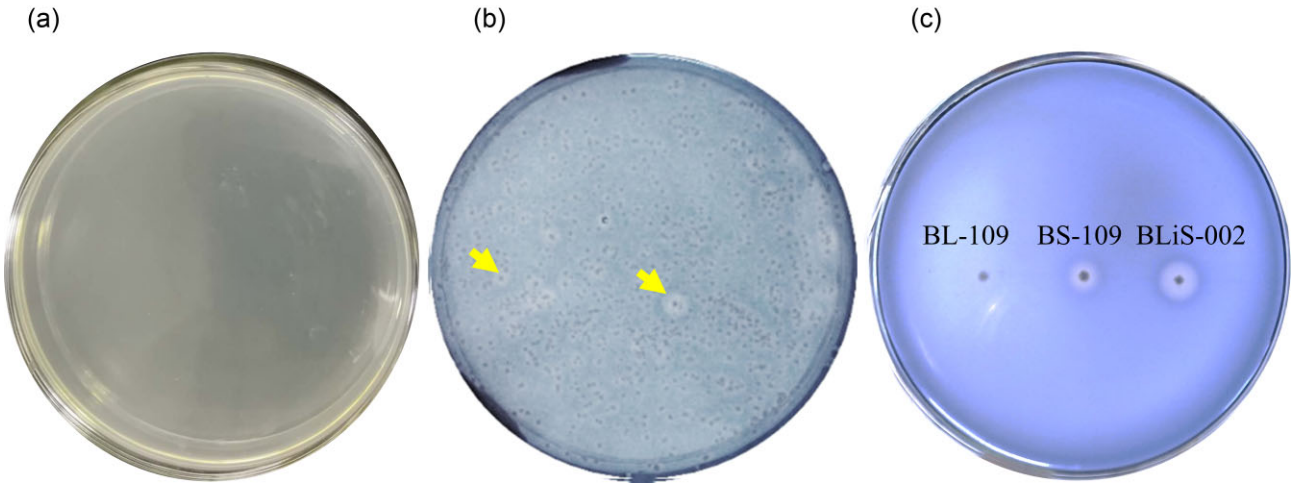

Fig. 5 The selection of target recombinants. The results of plating out the culture sample of BL-amyS on (a) LB plate containing 2 μg/ml tetracycline; (b) starch plate containing 500 μg/ml kanamycin; and (c) starch halo zone formed by *B. licheniformis* BL-109 (Δ*amyL*), BS-109 (*amyL*::*amyS*), and BLiS-002 harboring five *amyS* copies, on a nonselective starch plate. Arrows mark two of the target colonies with large starch hydrolysis zones as examples.

Assessment of Recombinant Stability and Enzyme Yield

The recombinants were subcultivated in nonselective LB medium for several cycles (cultivation was conducted at 37°C and 200 rpm for 12 hr) and plated out as single colonies on nonselective LB plates, and then colonies were picked and spotted on LB plates containing 20 μg/ml kanamycin and another nonselective LB plate, respectively. The fraction of colony-forming units that grew under restrictive conditions was measured to evaluate the genetic stability of recombinants. The first and 15th subcultures were used as seeds for flask fermentations, as described subsequently, to assess the enzyme expression stability (Song et al., 2017).

Flask fermentations were carried out as described in a previous study (Niu et al., 2009) with slight modifications. Briefly, a single colony of a recombinant strain was inoculated into LB medium and cultured overnight at 200 rpm and 37°C for 16 hr. Seed culture (5 ml) 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 hr.

A scaled-up fermentation was carried out in a 50-l fermenter to further evaluate the enzyme expression level of the selected recombinant. The fermentation conditions were described previously (Niu et al., 2009). The fermentation temperature was controlled at 42°C and the fermentation medium pH was controlled at 6.0 by feeding 25% (wt/vol) ammonium hydroxide automatically. The fermentation medium consisted of 40 g/l lactose, 25 g/l soybean meal, 20 g/l cottonseed meal, 30 g/l corn-steep liquor, and 0.01 mol/l ammonium sulphate. Culture samples were taken every 4 hr and used to measure enzyme activity and verify the genetic stability of recombinants through the halo zone formed on starch plates.

Enzyme Activity Assay and Other Analytic Procedures

The activity of the thermophilic α-amylase was determined as described by Hollo and Szeitli (Hollo & Szeitli, 1968). One unit of the enzyme was 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 in triplicate with an SP-2012UV spectrophotometer (Shanghai Spectrum Instruments, China). As the fermentation medium contained many particulates, the biomass was estimated by centrifugation of the fermentation broth. All data shown were means of at least three different experiments. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was adopted to analyze the protein profiles (Zhuge & Wang, 1994).

Results and Discussion

Helper Plasmid Curing and Nonreplicative Plasmid Integration in *B. licheniformis*

Very different from many other microorganisms, such as *E. coli*, *B. subtilis*, and *S. cerevisiae*, industrial strains of *B. licheniformis* are extremely difficult to genetically modify by direct transformation of free DNA fragments (Huff et al, 2017; Waschkau et al, 2008). Therefore, plasmid-mediated DNA transformation and integration is always used in the genetic manipulation of *B. licheniformis*. We tried to develop an α-amylase-overexpressing *B. licheniformis* strain according to the method described for *B. lentus* (Jørgensen et al., 2000). Consequently, we constructed two plasmids from pUB-EX (Fig. 1a): pUB-Tet (Fig. 1b) as a helper plasmid and pUB′-EX1 (Fig. 1c) as an integrative expression plasmid. This pair of plasmids were used to construct recombinant *B. licheniformis* for overexpressing an α-amylase (AmyS) from *G. stearothermophilus* ATCC 31 195. However, we failed to obtain the desired recombinants. The curing of the helper plasmid was inefficient in *B. licheniformis* when the incubation temperature was elevated, which led to inefficient screening of the expression plasmid integrated strains. The transformant harboring the two plasmids was cultivated in LB medium at 42°C and 200 rpm for 12 hr, then 2% of the inoculum was transferred to fresh LB medium and then repeated 4 times. After being incubated in nonselective LB liquid medium for four cycles, the culture was diluted and spread on nonselective LB plates. As shown in Fig. 2, the presence of plasmid pUB-Tet and pUB′-EX1 was evaluated on LB plates containing tetracycline (5 μg/ml) or kanamycin (20 μg/ml). Of 90 colonies, 86 colonies were found to be still resistant to tetracycline, which means that 96% of the colonies still harbored the helper plasmid after four cycles of subculture at 42°C. Furthermore, only one of the four "cured" colonies could grow on the LB plate containing 20 μg/ml kanamycin (the colony is circled in Fig. 2b), which means that the helper plasmid curing and integrative efficiency of pUB′-EX1 was extremely low. The possible

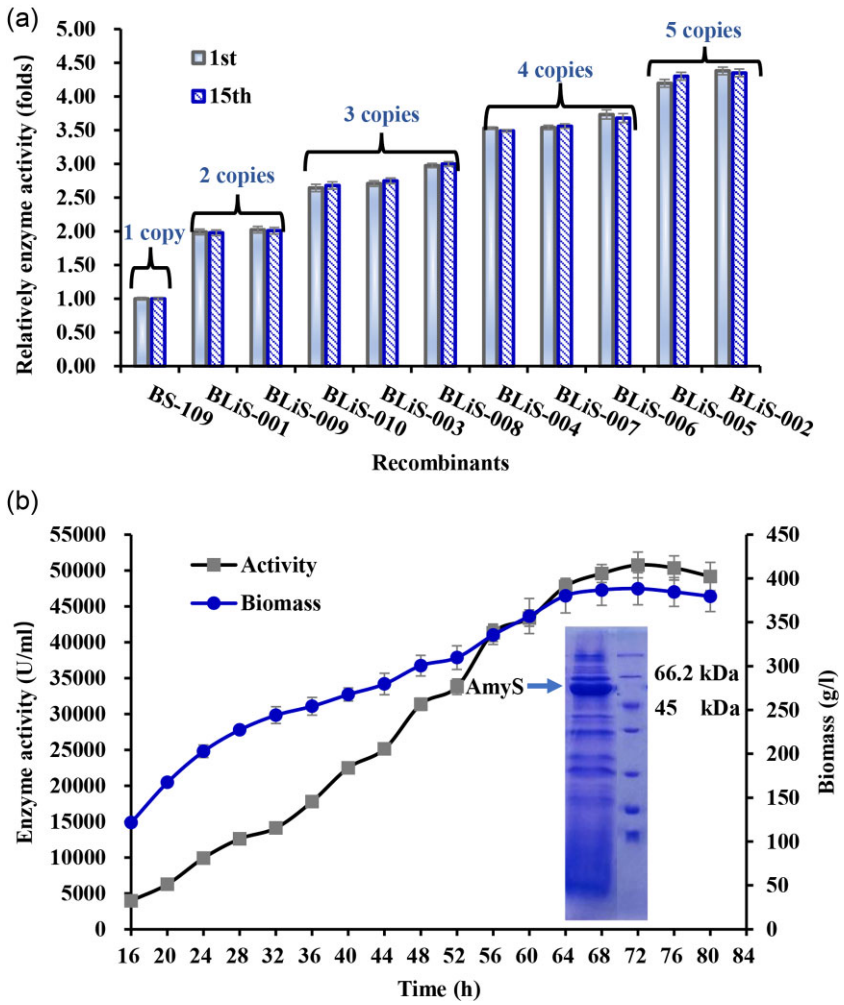

Fig. 6 Genetic stability, copy number of *amyS*, and fermentation performance of *B. licheniformis* recombinants. (a) The yields of α-amylase after 15 generations of 10 recombinant strains with different copy numbers of *amyS*. (b) The time course of AmyS production of recombinant *B. licheniformis* strain BLiS-002 in a 50-l fermenter; embedded box: SDS-PAGE profile of AmyS taken from the fermentation broth. Error bars indicate standard deviation from three parallel experiments.

reason is that we can only use the negative selection procedure to obtain the helper plasmid pUB-Tet-cured colonies. A high percentage of the noncured cells in the culture may significantly decrease the selection efficiency of cells integrated by the expression plasmid pUB′-EX1. However, the exact reason is unclear. The results strongly indicated that an alternative way to efficiently cure the helper plasmid should be found and that a new helper plasmid is required.

An RNase encoding gene *mazF* under the control of an IPTG-inducible promoter P_{spac} was inserted into the plasmid pUB-Tet, and a new replication-thermosensitive plasmid, pUB-MazF, was constructed (Fig. 1d).

Functional Verification of pUB-MazF and pUB′-EX1

The replication of pUB-MazF was verified in *B. subtilis* WB600 and *B. licheniformis* BL-109 (Fig. 3a), respectively. After 48 hr cultivation in LB medium without 1 mmol/l IPTG at 30 and 42°C, the recombinant *B. subtilis* and *B. licheniformis* harboring pUB-MazF grew well on LB plates with 15 and 2 μg/ml tetracycline, respectively. After 48 hr cultivation in LB medium with 1 mmol/l IPTG at 42°C, no recombinants grew on LB plates with tetracycline (Fig. 3a). These results indicated that plasmid pUB-MazF could be significantly cured by induction of MazF with IPTG.

The replication and integration of pUB′-EX1 with the assistance of pUB-MazF was verified in *B. subtilis* WB600 and *B. licheniformis* BL-109 (Fig. 3b). Both *B. subtilis* WB600 and *B. licheniformis* BL-109 harboring pUB-MazF and pUB′-EX1 were grown well on LB plates complemented with 2 μg/ml tetracycline and 20 μg/ml kanamycin at 30°C, which indicated that pUB-MazF assisted with the replication of pUB′-EX1 and both plasmids could be maintained and replicated in both hosts at lower incubation temperature. By incubating the strains in LB medium with 1 mmol/l IPTG at 42°C and 200 rpm for 48 hr, both strains grew well on LB plates with 20 μg/ml kanamycin but not on 2 μg/ml tetracycline plates (Fig. 3b). The results suggested that pUB′-EX1 was integrated into the chromosome with selection pressure (20 μg/ml kanamycin) and pUB-MazF was lost (curing) due to expression of MazF in pUB-MazF.

Overexpression of Thermophilic α-Amylase in *B. licheniformis* Using pUB-MazF and pUB′-EX1

Recombinant *B. licheniformis* strains overexpressing α-amylase were developed as described in the following steps (Fig. 4).

Step 1: pUB-MazF was transformed into *B. licheniformis* BL-109 by electroporation. After transformation, pUB-MazF was integrated into the chromosome of *B. licheniformis* BL-109 by raising the temperature to 42°C and selecting for resistance to tetracycline. The resulting recombinant was named as *B. licheniformis* BL-UBM. Step 2: the α-amylase integrative expression plasmid pUB′-amyS (Fig. 1e) was constructed and transferred into *B. licheniformis* BL-UBM by electroporation. The resulting transformant was named as BL-amyS. Production of α-amylase was predetected on starch plates containing 2 μg/ml tetracycline and 20 μg/ml kanamycin at 30°C. Step 3: BL-amyS was cultivated in LB medium with 500 μg/ml kanamycin and 1 mmol/l IPTG at 42°C for four subculture cycles, leading to the integrated fragment of helper plasmid pUB-MazF being cured from the chromosome and there being no more production of the replicase RepF. Consequently, pUB′-amyS was forced to integrate into the 3-*amyL*′ site of the *B. licheniformis* chromosome in the presence of 500 μg/ml kanamycin. The diluted culture samples were spread on LB plates containing 2 μg/ml tetracycline and starch plates containing 500 μg/ml kanamycin for confirmation of pUB-MazF curing and α-amylase activity, respectively (Fig. 5). No colony was grown on the LB plate containing 2 μg/ml tetracycline (Fig. 5a), which suggested that pUB-MazF curing was completed because any cell harboring pUB-MazF was killed by MazF expressed by inducing with IPTG. All colonies grown on the starch plate containing 500 μg/ml kanamycin had clear starch hydrolysis zones and many of them formed a larger halo zone (Fig. 5b). The integration of pUB′-amyS at the 3′-*amyL*′ site was further verified by colony PCR and the expected bands were obtained (Fig. S1). The colonies that showed different sizes of hydrolysis zones on starch plates and did not grow on LB plates containing 2 μg/ml tetracycline were picked up and nominated as BLiS-001–BLiS-010. The resulting recombinants shown significantly improved α-amylase activity (Fig. 5c). Compared with merely elevating the cultivation temperature (Fig. 2), the current strategy obviously improved the efficiency of helper plasmid curing due to employing the toxin protein MazF. The combination of pUB-MazF with pUB′-EX1 for introducing the heterologous gene into *B. licheniformis* is feasible.

The α-amylase productivity, genetic stability, and copy number of *amyS* of recombinants BLiS-001–BLiS-010 were analyzed using shaking flask fermentations and qPCR (Fig. 6a; Table S2). The enzyme productivity was directly proportional to the copy number of the gene when *amyS* copies increased from 1 to 3, while the productivity was not proportional to the copy number of *amyS* with further increased copies. Recombinant *B. licheniformis* BLiS-002 having five copies of *amyS* showed the highest enzyme activity and it was about 3.3-fold higher than the preconstructed strain BS-109 harboring one copy of *amyS*. The reason for the nonproportional relationship between the productivity and copy number of *amyS* at higher copies is that the enzyme productivity is also determined by many other factors, such as enzyme translocation efficiency, metabolic capacity, and ATP supply efficiency (Niu et al., 2009). All recombinants showed the desired genetic stability, and the enzyme productivities were almost unchanged after the 15th subculture (Fig. 6a; Table S3).

To further confirm the yield of α-amylase with the recombinant BLiS-002, large-scale fermentation was performed in a 50-l fermenter. The time course for AmyS production and cell growth of recombinant *B. licheniformis* strain BLiS-002 showed that enzyme activity and biomass increases were detected 16 hr after the start of the fermentation (Fig. 6b). The activity gradually increased up to 60 hr of incubation. The increase in activity slowed down between 60 and 80 hr. The maximum activity of α-amylase reached 50 753 U/ml after 72 hr of cultivation and then decreased slightly. After fermentation, the main protein in the broth was the expressed α-amylase in the SDS-PAGE profile (band at 55.5 kDa) (Fig. 6b). The yield of the α-amylase was ∼22-fold better than a previous report (Chen et al., 2015). The recombinant BLiS-002 showed perfect genetic stability and all the colonies of the recombinant BLiS-002 in fermentation broth could form a halo zone on starch plates after 80 hr fermentation (Fig. S2). By using this newly developed method, we have successfully and quickly overexpressed many other industrial enzymes, including pullulanase, lactase, bacterial α-amylase and alkaline protease in *B. licheniformis* (data not shown).

Conclusion

In this work, the strategy for integration of a heterologous enzyme-coding gene into the chromosome of a *Bacillus* sp. was developed through a pair of newly constructed plasmids, pUB-MazF as the helper plasmid and pUB′-EX1 as the integrative expression plasmid. The presence of *mazF* in pUB-MazF vastly facilitated the curing of pUB-MazF and the integration of pUB′-EX1. The strategy was successfully applied for the construction of enzymes overexpressing *B. licheniformis* strains with favorable genetic stability. This system may have a beneficial impact on industrial enzyme production in *B. licheniformis* and other *Bacillus* species.

Acknowledgments

We thank Dr Bernard A. Prior from Stellenbosch University and Dr Meng Zhang from Tianjin University of Science and Technology for their assistance in preparation of the manuscript.

Supplementary Material

Supplementary material is available online at *JIMB* (*www.academic.oup.com/jimb*).

Funding

This work was supported by the Intergovernmental International Scientific and Technological Innovation Cooperation Program, MOST, China (grant no. 2018YFE0100400) to W.Z.X., the Intergovernmental International Scientific and Technological Innovation Cooperation Program, MOST, China (grant no. 2021YFE0106200) to N.D.D., and the Tianjin Outstanding Talent Program (grant no. JC20200309) to W.Z.X.

Conflict of Interest

The authors declare no conflict of interest.

References

Al-Hawash, A. B., Zhang, X., & Ma, F. (2017). Strategies of codon optimization for high-level heterologous protein expression in microbial expression systems. *Gene Reports*, 9, 46–53. https://doi.org/10.1016/j.genrep.2017.08.006

Anagnostopoulos, C. & Spizizen, J. (1961). Requirements for transformation in *Bacillus subtilis*. *Journal of Bacteriology*, 81(5), 741–746. https://doi.org/10.1128/jb.81.5.741-746.1961

Bloor, A. E. & Cranenburgh, R. M. (2006). An efficient method of selectable marker gene excision by Xer recombination for gene replacement in bacterial chromosomes. *Applied and*

Environmental Microbiology, 72, 2520–2525. https://doi.org/10.1128/AEM.72.4.2520-2525.2006

Chen, J., Fu, G., Gai, Y., Zheng, P., Zhang, D., & Wen, J. (2015). Combinatorial Sec pathway analysis for improved heterologous protein secretion in *Bacillus subtilis*: Identification of bottlenecks by systematic gene overexpression. *Microbial Cell Factories*, 14, 92. https://doi.org/10.1186/s12934-015-0282-9

Dong, H. & Zhang, D. (2014). Current development in genetic engineering strategies of *Bacillus* species. *Microbial Cell Factories*, 13, 63. https://doi.org/10.1186/1475-2859-13-63

Hollo, J. & Szeitli, J. (1968). The reaction of starch with iodine. In J. A. Rodley (Ed.), *Starch and its derivatives* (pp. 203–246). Chapman & Hall.

Huff, F., Muth, C., Naumer, C., & Meinhardt, F. (2017). The restriction modification system of *Bacillus licheniformis* MS1 and generation of a readily transformable deletion mutant. *Applied Microbiology and Biotechnology*, 101, 7933–7944. https://doi.org/10.1007/s00253-017-8532-x

Ishiwa, H. & Shibahara, H. (1985). New shuttle vector for *Escherichia coli* and *Bacillus subtilis* II. Plasmid pHY300PLK, a multipurpose coning vector with a polylinker, derived from pHY460. *The Japanese Journal of Genetics*, 60, 235–243. https://doi.org/10.1266/jjg.60.235

Jørgensen, P. L., Tangney, M., Pedersen, P. E., Hastrup, S., Diderichsen, B., & Jørgensen, S. T. (2000). Cloning and sequencing of an alkaline protease gene from *Bacillus lentus* and amplification of the gene on the *B. lentus* chromosome by an improved technique. *Applied and Environmental Microbiology*, 66, 825–827. https://doi.org/10.1128/aem.66.2.825-827.2000

Li, K., Cai, D., Wang, Z., He, Z., & Chen, S. (2018). Development of an efficient genome editing tool in *Bacillus licheniformis* using CRISPR-Cas9 nickase. *Applied and Environmental Microbiology*, 84, e02608–17. https://doi.org/10.1128/AEM.02608-17

Li, Z., Li, Y., Gu, Z., Ding, Z., Zhang, L., Xu, S., & Shi, G. (2019). Development and verification of an FLP/*FRT* system for gene editing in *Bacillus licheniformis*. *Chinese Journal of Biotechnology*, 35, 458–471. https://doi.org/10.13345/j.cjb.180327

Macauley-Patrick, S., Fazenda, M. L., McNeil, B., & Harvey, L. M. (2005). Heterologous protein production using the *Pichia pastoris* expression system. *Yeast*, 22, 249–270. https://doi.org/10.1002/yea.1208

Morimoto, T., Ara, K., Ozaki, K., & Ogasawara, N. (2009). A new simple method to introduce marker-free deletions in the *Bacillus subtilis* genome. *Genes & Genetic Systems*, 84, 315–318. https://doi.org/10.1266/ggs.84.315

Nadler, F., Bracharz, F., & Kabisch, J. (2019). CopySwitch—*in vivo* optimization of gene copy numbers for heterologous gene expression in *Bacillus subtilis*. *Frontiers in Bioengineering and Biotechnology*, 6, 207. https://doi.org/10.3389/fbioe.2018.00207

Niu, D., Shi, G., & Wang, Z. X. (2009). Genetic improvement of α-amylase producing *Bacillus licheniformis* by homolog-mediated α-amylase gene amplification. *Chinese Journal of Biotechnology*, 25, 375–380.

Niu, D. & Wang, Z. X. (2007). Development of a pair of bifunctional expression vectors for *Escherichia coli* and *Bacillus licheniformis*. *Journal of Industrial Microbiology & Biotechnology*, 34, 357–362. https://doi.org/10.1007/s10295-007-0204-x

Niu, D., Zuo, Z., Shi, G. Y., & Wang, Z. X. (2009). High yield recombinant thermostable alpha-amylase production using an improved *Bacillus licheniformis* system. *Microbial Cell Factories*, 8, 58. https://doi.org/10.1186/1475-2859-8-58

Öztürk, S., Ergün, B. G., & Çalık, P. (2017). Double promoter expression systems for recombinant protein production by industrial microorganisms. *Applied Microbiology and Biotechnology*, 101, 7459–7475. https://doi.org/10.1007/s00253-017-8487-y

Sambrook, J. & Russel, D. W. (2001). *Molecular cloning: a laboratory manual*, (3rd edn). Cold Spring Harbor Press.

Schallmey, M., Singh, A., & Ward, O. P. (2004). Developments in the use of *Bacillus* species for industrial production. *Canadian Journal of Microbiology*, 50, 1–17. https://doi.org/10.1139/w03-076

Scorer, C. A., Clare, J. J., Mccombie, W. R., Romanos, M. A., & Sreekrishna, K. (1994). Rapid selection using G418 of high copy number transformants of *Pichia pastoris* for high-level foreign gene expression. *Biotechnology (N Y)*, 12, 181–184. https://doi.org/10.1038/nbt0294-181

Song, X., Liu, Q., Mao, J., Wu, Y., Li, Y., Gao, K., Zhang, X., Bai, Y., Xu, H., & Qiao, M. (2017). POT1-mediated δ-integration strategy for high-copy, stable expression of heterologous proteins in *Saccharomyces cerevisiae*. *FEMS Yeast Research*, 17, fox064. https://doi.org/10.1093/femsyr/fox064

Wang, Y., Liu, Y., Wang, Z., & Lu, F. (2014). Influence of promoter and signal peptide on the expression of pullulanase in *Bacillus subtilis*. *Biotechnology Letters*, 36, 1783–1789. https://doi.org/10.1007/s10529-014-1538-x

Waschkau, B., Waldeck, J., Wieland, S., Eichstädt, R., & Meinhardt, F. (2008). Generation of readily transformable *Bacillus licheniformis* mutants. *Applied Microbiology and Biotechnology*, 78, 181–188. https://doi.org/10.1007/s00253-007-1278-0

Xu, M., Ma, J. S., & Wang, Z. X. (2004). Effect of high osmolarity on electro-transformation efficiency of bacteria. *Journal of Wuxi University of Light Industry*, 23, 98–100.

Yano, K., Wada, T., Suzuki, S., Tagami, K., Matsumoto, T., Shiwa, Y., Ishige, T., Kawaguchi, Y., Masuda, K., Akanuma, G., Nanamiya, H., Niki, H., Yoshikawa, H., & Kawamura, F. (2013). Multiple rRNA operons are essential for efficient cell growth and sporulation as well as outgrowth in *Bacillus subtilis*. *Microbiology (Reading, England)*, 159, 2225–2236. https://doi.org/10.1099/mic.0.067025-0

Yan, X., Yu, H. J., Hong, Q., & Li, S. P. (2008). Cre/lox system and PCR-based genome engineering in *Bacillus subtilis*. *Applied and Environmental Microbiology*, 74, 5556–5562. https://doi.org/10.1128/AEM.01156-08

Young, M. & Ehrlich, S. D. (1989). Stability of reiterated sequences in the *Bacillus subtilis* chromosome. *Journal of Bacteriology*, 171, 2653–2656. https://doi.org/10.1128/jb.171.5.2653-2656.1989

Zakataeva, N. P., Nikitina, O. V., Gronskiy, S. V., Romanenkov, D. V., & Livshits, V. A. (2010). A simple method to introduce marker-free genetic modifications into the chromosome of naturally nontransformable *Bacillus amyloliquefaciens* strains. *Applied Microbiology and Biotechnology*, 85, 1201–1209. https://doi.org/10.1007/s00253-009-2276-1

Zhan, Y., Zhang, J., Hoeflich, K. P., Ikura, M., Qing, G., & Inouye, M. (2003). MazF cleaves cellular mRNAs specifically at ACA to block protein synthesis in *Escherichia coli*. *Molecular Cell*, 12, 913–923. https://doi.org/10.1016/s1097-2765(03)00402-7

Zhou, C., Liu, H., Yuan, F., Chai, H., Wang, H., Liu, F., Li, Y., Zhang, H., & Lu, F. (2019). Development and application of a CRISPR/Cas9 system for *Bacillus licheniformis* genome editing. *International Journal of Biological Macromolecules*, 122, 329–337. https://doi.org/10.1016/j.ijbiomac.2018.10.170

Zhuge, J. & Wang, Z. X. (1994). *Industrial microbiology. A laboratory manual*. China Light Industry Press.