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

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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 & Cranburgh, 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
loki 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

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**Table 1. Strains and Plasmids Used in This Study**

| Strains/Plasmids | Characteristics | Resource |
|------------------|-----------------|----------|
| E. coli JM109    | endA1, recA1, gyrA96, thi, hsdR17, relA1, supE44, λ−, Δlac-proAB, [F−, traD36, proAB, lacI2ΔZam15] | Lab stock |
| B. subtilis WB600| B. subtilis 168, ΔaprFL, ΔaprA, Δapr, Δhyf, Δmfr, ΔmprB | Lab stock |
| B. subtilis TM0310| aprE-spec, lac, p_MazF | Morimoto et al. (2009) |
| G. stearothermophilus ATCC 31915 | Host cell for gene expression | Lab stock |
| B. licheniformis CBBD302 | B. licheniformis CBBD302, amyl, | Niu et al., (2009b) |
| B. licheniformis BL-109 | B. licheniformis BL-109, amyl::amyS | This study |
| B. licheniformis BS-109 | Chromosome-integrated pUB-MazF in B. licheniformis BL-109 | This study |
| B. licheniformis BL-UBM | B. licheniformis BL-UBM, pUB-amyS | This study |
| B. licheniformis BL-amyS | B. licheniformis BL-109, two copies of 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, three copies of amyS | This study |
| B. licheniformis BLIS-003 | B. licheniformis BL-109, three copies of amyS | This study |
| B. licheniformis BLIS-008 | B. licheniformis BL-109, four copies of amyS | This study |
| B. licheniformis BLIS-010 | B. licheniformis BL-109, four copies of amyS | This study |
| B. licheniformis BLIS-004 | B. licheniformis BL-109, five copies of amyS | This study |
| B. licheniformis BLIS-006 | B. licheniformis BL-109, five copies of amyS | This study |
| B. licheniformis BLIS-007 | B. licheniformis BL-109, five 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 |
| pHY300PK | TetR, ApR, E. coli–Bacillus sp. shuttle plasmid | Ishiwa & Shibahara, (1985) |
| pHY-WZX | TetR, ApR, KmR, expression vector | Niu & Wang (2007) |
| pUB-EX | KmR, thermosensitive plasmid, harboring the expression cassette of pHY-WZX | Lab stock |
| pUB-Tet | This study |
| pUB-MazF | TetR, derived from pUB-EX | This study |
| pUB-sint | TetR, replication-thermosensitive E. coli–Bacillus sp. shuttle vector, carrying pMazF controlled the transcription of mazF | This study |
| pUB-EX1 | KmR, ΔrepF, derived from pUB-EX | This study |
| pUB-amyS | KmR, integrative vector, thermosensitive and nonreplicable independent plasmid in Bacillus sp. | This study |
| pUB-amyS | KmR, integrated expression plasmid, pUB-EX1 carrying α-amylase expression cassette amyS from G. stearothermophilus ATCC 31195 | This study |
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 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 Bacillus licheniformis CBBD302 chromosome using primers P9 and P10. It was inserted into the BamHI 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 Bacillus subtilis TMO310 by PCR amplification using primers P7 and P8 and cloned into the SmaI site of pUB-Tet to obtain helper plasmid pUB-MazF (d). The amyS encoding the thermophilic α-amylase from G. stearothermophilus ATCC 51195 was isolated by PCR amplification with primers P11 and P12, and the PCR product was cloned into the XbaI and SmaI sites of pUB′-EX1 after being digested by XbaI, yielding α-amylase integrative expression plasmid pUB′-amyS (e). The elements in the vectors as follows: repF, encoding a thermostable replicase, ΔrepF, represents the nonfunctional thermostable replicase gene sequence; PamyL, SamyL, and T αT 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 Bacillus 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 Bacillus subtilis TMO310 (Morimoto et al., 2009). Bacillus licheniformis BL-109 derived from Bacillus 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.
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_{lac} 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 Anagnostopoulou & Spizizen (1961). Genetic transformation of B. licheniformis CBBD302 was done by electroporation 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 37°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 37 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 plates.

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**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.
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^ΔΔCt method as described previously [Niu et al., 2009], using *B. licheniformis* BS-109 with one copy of *amyS* as the control.
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 spots 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 Szeiti (Hollo & Szeiti, 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 31195. 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 (10 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
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_gal 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′-amylL 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′-amylL 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 pullulase, 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.academicoup.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.

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