生物工程学报
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Chin J Biotech 2009, March 25; 25(3): 375-380
Chinese Journal of Biotechnology ISSN 1000-3061

工业生物技术

通过同源介导α-淀粉酶基因扩增改良地衣芽孢杆菌α-淀粉酶生产菌株

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摘　要: 地衣芽孢杆菌高温α-淀粉酶(BLA)是淀粉水解与生物加工过程中重要关键酶制剂之一。为了进一步提高地衣芽孢杆菌高温α-淀粉酶生产菌株的生产性能, 本研究构建了一种含有地衣芽孢杆菌高温α-淀粉酶编码基因 *amyL* 的整合性重组质粒 pBL-amyL。将重组质粒 pBL-amyL 转化入 BLA 工业生产菌株 *Bacillus licheniformis* B0204, 再在卡那霉素存在下介导其 *B. licheniformis* B0204 染色体中的同源整合与高温α-淀粉酶编码基因 *amyL* 的扩增, 由此获得了携带多个 *amyL* 拷贝的转化子。对转化子的 *amyL* 拷贝数及其 BLA 发酵水平分别用荧光实时定量 PCR 及摇瓶发酵试验进行评价与鉴定。与出发菌株 B0204 相比, 含 2~5 倍 *amyL* 拷贝数的重组菌的 BLA 的合成水平显著提高。其中, 重组菌 REBL18 生产 BLA 的水平提高了 89.2%。

关键词: 地衣芽孢杆菌α-淀粉酶, 基因扩增, 发酵

Genetic improvement of α-amylase producing *Bacillus licheniformis* by homolog-mediated α-amylase gene amplification

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Abstract: *Bacillus licheniformis* α-amylase (BLA) is one of the most important enzymes involved in starch hydrolysis and many biotechnological processes. To improve the BLA productivity, an integrative plasmid pBL-amyL carrying *amyL* gene encoding a thermophilic α-amylase of *B. licheniformis* was constructed and transformed into *B. licheniformis* B0204, an industrial α-amylase producer. The transformants harboring different copies of *amyL* were developed on kanamycin by using homolog-mediated chromosomal amplification of α-amylase gene. The recombinants with different multiple copies of *amyL* integrated in the chromosome were identified by real-time PCR and evaluated by shake-flask fermentation. Recombinants harboring 2−5 multiple copies of *amyL* produced more α-amylase comparison to the parental strain B0204.

Keywords: *Bacillus licheniformis* α-amylase, gene amplification, fermentation

Received: October 23, 2008; **Accepted:** December 30, 2008
Supported by: National High-tech Research and Development Program of China (No. 2006AA020204).
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国家高技术研究发展计划(No. 2006AA020204)资助。

α-amylase (EC 3.2.1.1) is an endo-1,4-α-D-gluco-hydrolytic enzyme, which occurs widely in microorganisms, plants and animals. α-amylase and the related amylolytic enzymes are widely used in biotechnology for starch degradation[1] and in synthetic chemistry for the formation of oligosaccharides by transglycosylation[2]. One of major applications of the enzyme is to liquefy starch for the production of glucose and high-fructose corn syrup. A mesophilic strain of *Bacillus licheniformis* is commonly used worldwide to produce thousands tons of *Bacillus licheniformis* α-amylase (BLA)[3].

B. licheniformis is a mesophilic, spore-forming organism but produces a highly thermostable α-amylase[4]. The native strain does not yield significant amounts of α-amylase which indicates the need for genetic improvements of the strain. Traditional strain improvements and process optimization strategies have been performed intensively and have resulted in significant improvement of the yield of α-amylase by *B. licheniformis*. For example, the yield and fermentation performance have been dramatically improved by using sporulation-deficient and protease-deficient mutants obtained by physical or chemical mutagenesis[5]. However, few reports have focused on the molecular improvement of BLA yield in *B. licheniformis*, especially in BLA overproducers developed by the traditional mutagenesis techniques. In the study, we report that the yield of BLA has been significantly improved by the chromosomal amplification of *B. licheniformis amyL* in *B. licheniformis* B0204.

1 Materials and methods

1.1 Plasmids, bacteria and cultivation conditions

Escherichia coli XL1 used as host cell was cultivated at 37°C in Luria–Bertani medium (LB). As required, 100 μg/mL ampicillin and/or 25 μg/mL kanamycin were added to the medium. *B. licheniformis* B0204, a sporulation-deficient industrial strain for BLA preparation developed by intensive physical and chemical mutagenesis, was purchased from CICIM-CU (http://cicim-cu.jiangnan.edu.cn) and cultivated at 42°C in LB. For the shake-flask fermentation evaluation, the fermentation medium (FM) composed of 30 g/L corn-steep liquor, 30 g/L ammonium nitrate and 40 g/L lactose, pH 7.0, was used. Plasmid pBL-WZX[6] was used as an integrated expression vector in *B. licheniformis* B0204.

1.2 Construction of expression vector pBL-amyL

Polymerase chain reaction (PCR) was carried out with *Pfu* DNA polymerase (Fermentas, China) at an annealing temperature of 56°C. A 1.6 kb fragment containing *amyL* coding for the mature peptide as well as its 180 bp downstream sequence was amplified with primers F2-*Eco*R I (5′-CGGAATTCCTTAATGGGAC GCTGATGC-3′) and R1-*Sma* I (5′-TACCCGGGTACA TCAGATAACGTTGCC-3′) using pET28a-amyL$_{NEW}$[7] as template. The amplified product was purified and digested with *Eco*R I and *Sma* I and subsequently cloned into the same sites of pBL-WZX[7] to yield recombinant pBL-amyL (Fig. 1). Nucleotide sequencing of *amyL* was carried out by the dideoxy chain termination method using a DNA sequencing kit-BigDye terminator cycle sequencing ready reaction (Applied Biosystems) and an automated DNA sequencer (ABI 3130).

1.3 Transformation of *B. licheniformis* and identification of the recombinants

B. licheniformis B0204 cells were electrotransformed with pBL-amyL DNA according to the methods described previously[8]. The transformation mixture was cultivated and selected on LB medium with 5 μg/mL kanamycin at 42°C for 2 days. The gene amplification in the chromosome of *B. licheniformis* B0204 was induced by cultivating the transformants on the medium containing 10–25 μg/mL of kamamycin. Subsequently, shake-flask fermentation evaluation of the transformants was carried out at 42°C and 220 r/min for up to 144 h in a 250 mL flask with a working volume of 50 mL and the amylase activities were determined in every 4 hours.

The copies of the integrated *amyL* gene in the chromosome were analyzed by quantitative real-time PCR. SYBR Green I (Shinegene, China) was used as fluorescence dye and real-time PCR reactions were performed in 0.2 mL tubes with a MJ Research PTC-200 Peltier Thermal Cycle (Bio-Rad). All reactions were run in triplicate. $2^{-\Delta Ct}$ method was used to analyze relative gene multiples according to Livak and Schmittgen[9].

1.4 Enzyme assay

The activity of α-amylase was determined according to Chinese State-Standard QB/T 2306-97. One unit of the enzyme activity is defined as the amount of enzyme that hydrolyzes 1 mg water soluble corn starch per minute at 70°C and pH 6.0.

1.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The fermentation supernatant was analyzed by SDS-PAGE within a 10.0% polyacrylamide gel and stained with Coomassie Brilliant Blue G-250.

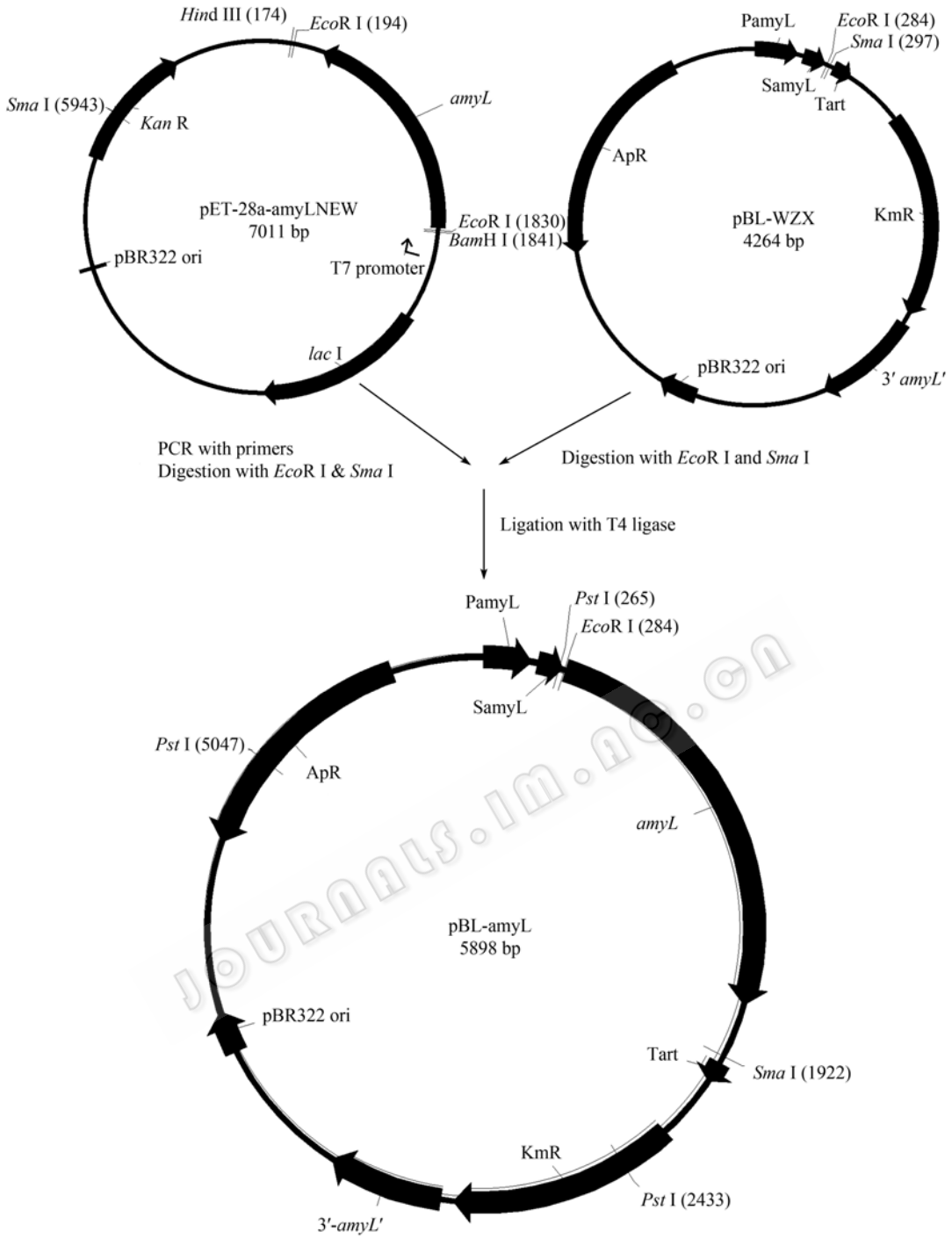

Fig. 1 The flowchart of pBL-amyL construction. A 1.6 kb DNA fragment containing *amyL* coding for the mature peptide was amplified and cloned into pBL-WZX to yield recombinant plasmid pBL-amyL. P_{amyL}: promoter of *B. licheniformis* B0204 *amyL*; S_{amyL}: signal encoding sequence of *B. licheniformis* B0204 *amyL*; T_{art}: an artificial terminator; 3'-*amyL*': 0.41 kb fragment containing 3'-terminal of *B. licheniformis* B0204 *amyL* and its downstream sequence.

2 Results

2.1 Construction of a recombinant plasmid for homolog-mediated chromosomal amplification of α-amylase gene

A 1.6 kb fragment containing *B. licheniformis* B0204 *amyL* coding for the mature peptide of BLA was cloned into pET28a to yield a recombinant plasmid pET28a-amyL$_{NEW}$. The recombinant plasmid was confirmed by nucleotide sequencing and BLA expression in *E. coli*[7]. To genetically improve the yield of thermostable α-amylase, a recombinant

plasmid for homolog-mediated chromosomal amplification of α-amylase gene in *B. licheniformis* was constructed as follows: A 1.6 kb fragment containing *B. licheniformis* B0204 *amyL* coding for the mature peptide of BLA was recovered from pET28a-amyL$_{NEW}$ by PCR. The *Eco*R I and *Sma* I digested PCR product was then subcloned into the same sites of pBL-WZX, resulting in pBL-amyL (Fig. 1).

The recombinant plasmid was confirmed by restriction analysis and nucleotide sequencing. A 412 bp 3′-*amyL*′ fragment was introduced into pBL-amyL and followed by a kanamycin selection cassette, which can mediate the integration amplification of the *amyL* in *B. lichenformis* chromosome[6].

2.2 Increase of *amyL* copies is benefit for host to synthesize more α-amylase

The recombinant plasmid pBL-amyL was transformed into *B. licheniformis* B0204 by electrotransformation. The transformation mixture was selected on LB plates containing kanamycin. Thrirty-six transformants were identified by the presence of enlarged halo on a corn starch agar plates when compared to the parent strain. All the transformants showed an increase in amylase activity in shake-flask fermentation experiments (data not shown). To induce the chromosome-amplification of *amyL*, one of above transformants, nominated as rBL01, was further cultivated on a medium containing up to 25 μg/mL kanamycin. The kanamycin-induced cells were streaked out to obtain single colonies. Fifty colonies (designated as REBL01 to REBL50) were randomly selected to determine the yields of α-amylase activity by shake flask evaluation in triplicate. Eighty-four percent of the colonies revealed increased yields of α-amylase with the percent increase ranging from 10% to more than 70%. Twelve of the colonies produced 70% greater α-amylase activity than the parent strain suggesting that an increase in *amyL* copies could result in greater amounts of α-amylase synthesis in the host.

2.3 An optimal copy number of *amyL* existed for host cells

To demonstrate the relationship between the copy number of *amyL* and the yield of the enzyme, four representative and genetically stable transformants, REBL06, REBL09, REBL18 and REBL43, were further examined for their enzyme yield by shake flask fermentation and their copy numbers of *amyL* by real-time PCR. Compared with that of the parental strain under the same growth conditions, the enzyme yield of the transformants increased significantly while the copies of *amyL* ranged from 2 to 5 (Table 1). Furthermore, an optimal integrated copy number of 2–3 of *amyL* for the maximum yield of α-amylase in *B. licheniformis* B0204 was apparent (Table 1).

Table 1 The relationship between amylase activity and *amyL* copy numbers

Strain[a]	Relative activity (%)	Copies of amyl (C_t)[b]	Multiples of amyl ($2^{-\Delta C_t}$)[b]
B0204	100.0	18.96±0.026	1.00
rBL01	132.3	17.98±0.074	1.98±0.093
REBL06	162.0	17.70±0.111	2.40±0.174
REBL18	189.2	17.39±0.006	2.98±0.024
REBL43	124.7	16.79±0.087	4.52±0.270
REBL09	32.1	15.75±0.124	9.28±0.857

[a] All strains were grown in 250 mL shake flasks with the working volume of 50 mL and incubated at 220 r/min for 144 h.

[b] $2^{-\Delta C_t}$ method was used to analyze relative gene multiples. The threshold cycle (C_t) is the PCR cycle at which fluorescence exceeds background and a significant increase in fluorescence is observed. A lower C_t value implies a higher starting quantity of the nucleic acid target. All data are averages of triplicate determinations.

2.4 α-Amylase production by recombinants

A strain with a multiple copy of a gene may increase production of its product or cause an intracellular physiological disorder, thereby affecting cell growth and metabolism. To reveal the reason for the variation in α-amylase yield by recombinants, REBL06, REBL09, REBL18 and REBL43 as well as parental strain B0204, the strains were evaluated in medium FM for cell growth and the enzyme production (Fig. 2, Fig. 3). All recombinants performed a slower growth rate than parental strain B0204. Among them, recombinant REBL06 and REBL18, carrying 2~3 copies of *amyL*, showed an acceptable growth rate and higher enzyme yield. Recombinant REBL43, carrying more than 4 copies of *amyL*, showed a slight increased enzyme yield but its growth was much slower than the parent strain. On the other hand, recombinant REBL09, carrying about 9 copies of *amyL*, showed the slowest growth and a decreased enzyme yield over the evaluation time. The protein yield of the recombinants (Fig. 3) was related to the enzyme activity (Table 1). This observation indicated that the lower enzyme activity found in recombinant REBL09 was not caused by inactivation of the enzyme but rather by a reduction in enzyme secretion. Similarly the increased enzyme activity found in recombinant REBL18 was directly linked to the enzyme yield.

Spore-formation may cause a termination of cell growth and fermentation, hence terminates the enzyme production. To reveal if there exists a reverse mutation

in spore formation in transformant REBL09, the spore formation was microscopically examined. Over the 120 h cultivation period, the spore formation rate was less than 1%, which was not different from parental strain B0204. When the fermentation evaluation was extended up to 240 h, transformant REBL09 produced a similar amount of the enzyme (data not shown) to that of parental strain B0204. These findings indicate that the lowest growth rate is a main reason for its lower enzyme yield although transformant REBL09 carried more copies of *amyL*.

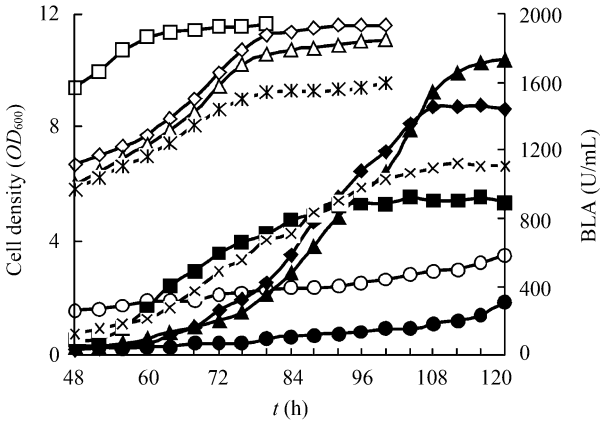

Fig. 2 BLA production from *B. licheniformis* carrying multiple copies of *amyL*. Cell growths of B0204 (□), REBL06 (◊), REBL09 (○), REBL18 (Δ) and REBL43 (*) are presented with optical density at 600 nm. BLA production from B0204 (■), REBL06 (♦), REBL09 (●), REBL18 (▲) and REBL43 (×) are presented with activity unit per milliliter.

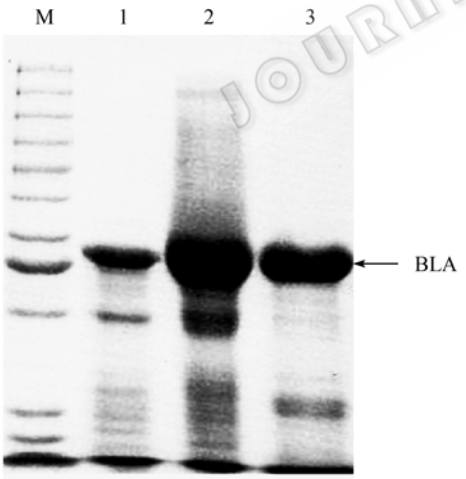

Fig. 3 SDS-PAGE profile of the supernatants by recombinants, REBL09, REBL18 and parental strain B0204. M: molecular weight marker (from up to down: 200, 150, 120, 100, 85, 70, 60, 50, 40, 30, 25 bp); 1: recombinant REBL09; 2: recombinant REBL18; 3: parental strain B0204.

3 Discussion

To improve the productivity of an enzyme, additional copies of its coding gene can be introduced either by genetic recombination or retransformation. For example, Srinivasan *et al* [10] described the production of recombinant proteins using multiple-copy gene integration in *Ralstonia eutropha*, in which organophosphohydrolase yield was increased approximately 30 times. Similarly by increasing copies of *glaA* in an industrial glucoamylase producer, 24.5% more enzyme activity could be produced[11]. In this study, an industrial strain of *B. licheniformis* carrying multiple *amyL* copies in the chromosome was constructed by homolog-mediated amplification. The production of α-amylase was further improved (Fig. 2, Fig. 3).

Theoretically, the amount of enzyme produced is linearly proportional to the gene copy number or the number and half-decay of the functional mRNA molecules. Furthermore, several other factors may also affect on the yield of the enzyme. These may include regulation of transcription, efficiency of translation, peptide folding and degradation, secretory machineries as well as cell global regulation and energy regeneration. Therefore, without modification of the associated physiological properties, a certain host cell can normally perform its highest productivity of an enzyme under an optimal gene copy number. In this study, 2–3 multiple copies of *amyL* in the chromosome was found to be optimal for higher expression of α-amylase in *B. licheniformis* B0204 (Table 1, Fig. 2, Fig. 3). On the other hand, more copies of a desirable gene in the chromosome, as shown in present study where *amyL* in REBL09 was found to exist in more than 9 copies in the chromosome (Table 1), might cause a disorder in transcription, translation, peptide folding or secretion in the host, resulting in the poor yield of the enzyme in a defined time, as shown by recombinant REBL09 (Fig. 2, Fig. 3).

B. licheniformis may hold a specific capacity maximum for protein secretion. Beyond the maximum capacity, introducing more copies of a gene may result in a shortage in energy and metabolites or a disorder in protein secretion machinery. It is reported that *B. licheniformis* is able to secret as much as 25 mg/mL of a specific protein[12]. The recombinant REBL18 was tested in a 30 ton fermentor, the enzyme activity reached about 14 600 U/mL, which was about 15 mg/mL of active form of BLA (unpublished data). This further raises an interesting question whether the yield of a beneficial enzyme may be subsequently increased if the physiological properties of a host cell are genetically improved to be suitable for expression of multiple copies of a gene[13].

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