

Overproduction of *Rummeliibacillus pycnus* arginase with multi-copy insertion of the *arg^{R.pyc}* cassette into the *Bacillus subtilis* chromosome

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Abstract A plasmid-less and marker-less strain with multi-copy integration of the arginase gene from *Rummeliibacillus pycnus* was constructed using *Bacillus subtilis* 168 as a host. A total of nine copies of the *arg^{R.pyc}* cassettes, in which the *R. pycnus* arginase gene was fused with the strong promoter *P43*, were inserted into the recipient chromosome. These multiple insertions were completed via step-by-step integrations into designed (2 copies) and random (9 copies) sites, respectively. A strategy for random site integration was developed based on the construction of the *arg^{R.pyc}* cassette sandwiched between “front” and “back” homologous arms which were randomly restricted from chromosomal DNA. An antibiotic resistance marker was applied in transformant selection and was eliminated via the *Cre/lox* system. Performance showed that the highest enzyme activity (14.5 U/mL) was obtained after culture in flasks, and this segregation stable strain could efficiently hydrolyze L-arginine with a 97.2% molar yield, showing potential application in the food industry.

Keywords L-ornithine · *Bacillus subtilis* · *Rummeliibacillus pycnus* arginase · Integration · Multi-copy · Marker-less

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Introduction

L-Ornithine is a non-essential amino acid that plays important roles in metabolism. This amino acid is a drug component for mediating liver protection and treating liver disease (Jalan et al. 2007). L-Ornithine is an ideal nutritional supplement for athletes and bodybuilders, and this amino acid is effective for both relieving stress and improving sleep (Sugino et al. 2008). Therefore, great demand exists for L-ornithine in the food supplement and pharmaceutical industries. For these reasons, a simple method for L-ornithine production is needed.

Arginase (L-arginine amidohydrolase, EC 3.5.3.1) is a binuclear metalloenzyme that hydrolyzes L-arginine to L-ornithine and urea. Compared with other methods, the use of arginase hydrolysis has several advantages, including the independence on expensive equipment, specificity for substrate, and rapid production (Song et al. 2014). Currently, various arginases from different resources have been overexpressed in *Escherichia coli* (Song et al. 2014; Yu et al. 2013). Although these recombinant enzymes or cells showed L-ornithine biosynthesis abilities, the safety of engineered microorganisms and their products in the food industry presents growing concern by the public (He et al. 2016).

It is revealed that more than one-third of the industrial enzymes are produced by *Bacillus* strains (Meissner et al. 2015). *Bacillus subtilis*, regarded as the “Generally Recognized as Safe” (GRAS) organism for its lack of endotoxins and pathogenicity, has a long history of industrial use (Jeong et al. 1998; Sietske and Diderichsen 1991). Traditional methods of genetically engineering *B. subtilis* strains involve the introduction of a recombinant plasmid (Povelainen and Miasnikov 2007). However, this method has limitations, such as instability and usage of antibiotic resistance markers (Fleming and Patching 1994; Kastner et al. 2006). Chromosome integration may be an excellent way to

overcome the disadvantages of the plasmids and remove the restrictions on the use of plasmid strains in industry. Usually, homologous recombination with the integration of target gene into desired chromosomal regions is applied in plasmid-less strain construction (Sauer et al. 2016). In addition, transposition and site-specific recombination are also utilized for the same integration strategy.

Homologous recombination can be performed through Campbell-type single-crossover recombination using circularized DNA plasmids with internal homologous fragments or through the use of double-crossover recombination with the target gene sandwiched by homologous flanking sequences (Yomantas et al. 2011). However, single-crossover recombination is unstable under non-selective conditions for the possible elimination of the inserted plasmid (Lee et al. 2016). Because of the low efficiency of single-crossover recombination, gene replacement by double-crossover has been designed for target gene integrations. Ectopic insertion with double-crossover leads to more stable recombinant strains.

With the increase of insertion DNA cassette copy number, high production of proteins could be obtained (Zhu et al. 2009). Several methods are available for multi-copy insertions of the target gene. One method uses PCR or isocaudamer enzymatic polymerization technology to construct tandem repeat sequences which are then integrated into target chromosome regions (He et al. 2016). The other one is using PCR or available plasmids to construct a set of DNA cassettes, which contain different homologous fragments and are then integrated into different chromosome regions with several steps (Yomantas et al. 2011). Amplification of the same gene in chromosomal DNA would certainly decrease the stability of the strains because of the potential of homologous chromosomal recombination. Directly repeated cassette insertion in one site would lead to internal chromosome deletions or loss by cyclization (Bron et al. 1991). In turn, integration in different sites may be more stable.

Previously, a thermostable arginase from *Rummeliibacillus pycnus* SK 31.001 was found and its gene was sequenced (Huang et al. 2016). Characterization of the enzyme revealed that this enzyme could be a good candidate for L-ornithine biosynthesis. In this study, a recombinant plasmid-less and antibiotic resistance marker-less *B. subtilis* strain was constructed which can efficiently produce *R. pycnus* arginase. To improve the expression level of *R. pycnus* arginase, the arginase gene was fused with a *P43* strong promoter to generate a *P43-arg* (*arg^{R.pyc}*) expression cassette. Initially, the *argI* gene encoding *B. subtilis* arginase was knocked out to avoid the effects of this protein. Then, two copies of the expression cassettes were integrated into two designed regions in *B. subtilis* chromosome, respectively. Combined with the random integration method, a recombinant strain with nine copies of insertion cassettes was obtained. An antibiotic resistance marker was applied in transformant selection and was

eliminated via the *Cre/lox* system. The performance of the new multi-copy strain showed that this marker-less and segregation stable recombinant strain could be a food-grade candidate for L-ornithine biosynthesis.

Materials and methods

Strains, plasmids, materials, and media

Strains and plasmids used in this study were listed in Table S1. All recombinant *Bacillus* strains were derived from *B. subtilis* 168.

R. pycnus was cultured in the medium which contained the following: 1% (w/v) glucose, 0.5% (w/v) peptone, 0.5% (w/v) yeast extract, 0.1% (w/v) K₂HPO₄, pH 6.5, 37 °C. *E. coli* and *B. subtilis* strains were grown at 37 °C in Luria Bertani (LB) medium (1% (w/v) NaCl, 1% Trypton, 0.5% (w/v) yeast extract, 1% (w/v) peptone) or LB with agar supplemented by antibiotics (spectinomycin (Spc, 100 mg/L), zeocin (Zeo 20 mg/L), or ampicillin (Amp, 100 mg/L)) when needed. Successful insertions of the *arg^{R.pyc}* cassettes were confirmed by inoculation of recombinant cells on LB agar plates (containing 0.002% (w/v) phenol red, pH 6.0).

Seed cultures were prepared with inoculating colonies into 25-mL LB media in 250-mL flasks, and then 3% (v/v) seed cultures were inoculated into another 25-mL fresh LB medium for fermentation at 200-rpm rotary shaker at 37 °C.

Genetic engineering methods

Spizizen method was used for transformation of *B. subtilis* (Anagnostopoulos and Spizizen 1961).

Taq DNA polymerase, PrimeSTAR Max DNA polymerase, restriction endonuclease, SYBR Premix EX *Taq*TM II, and TA cloning vectors were purchased from TAKARA Co., Ltd. (Dalian, China). T4 DNA ligase, mini chromosome rapid isolation kit, DNA gel extraction kit, mini plasmid rapid isolation kit, oligonucleotide synthesis, and direct sequencing were accomplished by Sangon Biotech Co., Ltd. (Shanghai, China).

All the primers were listed in Table S2.

Enzyme activity assay

The arginase activity assay was determined using L-arginine as substrate. The reaction was performed in 50 mM NaOH-glycine buffer (pH 9.5) with 100 mM L-arginine and 1 mM Mn²⁺. The reaction was started by adding 200 μL fermentation broth to 1 mL reaction mixture at 80 °C. After incubation for 10 min, reaction was terminated with 200 μL 1.5 M trichloroacetic acid (TCA). Reaction without substrate added was taken as blank. Produced L-ornithine was measured by

using HPLC (Zhang et al. 2013). One unit of arginase activity was defined as the amount of enzyme required to generate 1 μmol of L-ornithine per minute under the conditions above. Arginase activity was expressed as units per milliliter sample.

Construction of the BSKA-A2 ($\Delta amyE$, $\Delta thrC::arg^{R.pyc}$) strain

To eliminate any effect of the arginase from *B. subtilis* 168, the arginase gene of *B. subtilis* 168 (*argI*) was knocked out. The *Zeo^R* gene with two *lox*-sites (7Z6: *lox72-zeo-lox66*) and homologous arms were amplified from plasmid p7Z6 (primers—P1/P2). “Front” and “back” homologous arms (*argIF*, *argIB*) were amplified from the *B. subtilis* chromosome (primers—P3/P4, P5/P6). The linear DNA fragment, *argIF*-7Z6-*argIB*, was constructed with overlapping PCR and used for *argI* deletion via double-crossover recombination (shown in Fig. S1). Competent strains were selected on LB agar containing zeocin (100 mg/L) and then transformed with the plasmid pTSC. The *zeo* resistance gene could be deleted via the *Cre/lox* system (recombination between *lox71* and *lox66* by *Cre* recombinase) (Yan et al. 2008). For elimination of pTSC, transformants were patched on LB agar without antibiotic and incubated at 51 °C. The resulting strain colonies lost pTSC yielding the BSKA strain.

The linear DNA fragment, *arg^{R.pyc}*-7S6 (7S6: *lox72-spc-lox66*), used for integration into the *B. subtilis* chromosome was constructed with overlapping PCR (shown in Fig. S1) and then sandwiched by “front” and “back” homologous arms. *P43*, arginase gene, and *Spc^R* gene with two *lox*-sites were amplified from *B. subtilis* chromosome, *R. pycnus* chromosome, and plasmid p7S6 (primers—P7/P8, P9/P10, P11/P12), respectively. The BSKA-A2 ($\Delta amyE$, $\Delta thrC::arg^{R.pyc}$) strain was completed via step-by-step insertion of two *arg^{R.pyc}* cassettes into the *amyE* and *thrC* genes of the BSKA strain. The overall scheme for the *arg^{R.pyc}* cassette integration is shown in Fig. 1 (where the insertion of the *arg^{R.pyc}* cassette into *B. subtilis amyE* was taken as an example). First, a linear DNA fragment, the *arg^{R.pyc}* cassette ligated with a resistance selection marker, was flanked with homologous arms. Second, linear DNA fragments were integrated into the corresponding chromosomal region via double-crossover recombination. Finally, the resistance marker cassette was eliminated by the *Cre/lox* system leaving a 34-bp *lox72* site in the chromosome. This step was followed by plasmid pTSC curing and led to construction of a marker-less and plasmid-less *R. pycnus* arginase-producing strain. The constructed strain was used as the recipient for the next integration using the same method. Step-by-step ectopic insertion resulted in the desired BSKA-A2 ($\Delta amyE$, $\Delta thrC::arg^{R.pyc}$) strain with 2 integrated *arg^{R.pyc}* cassettes.

Random integration of the *arg^{R.pyc}* cassette

The random integration was based on the competent strain of BSKA. A total of 10 μg BSKA chromosomal DNA were digested by *Bam*HI followed by self-circularization with T4 ligase. The reaction mixture was then digested by *Eco*RI, and the products were purified by agarose extraction. Purified DNA products (5 μg) were then ligated with the DNA fragments of *Eco*RI-generated *arg^{R.pyc}*-7S6 (5 μg), which was amplified from the linear DNA fragment *arg^{R.pyc}*-7S6 constructed above (primers—P21/P22). The ligation mixture was digested by *Bam*HI, and the digested products were used for transformation of BSKA. Finally, N copies of the *arg^{R.pyc}* cassettes were integrated into the chromosomal regions.

Segregation stability test

Spc^R recombinant strains

More than 100 individual colonies of recombinant strains were tested for *Spc^R*. Cells were plated on an LB agar plate containing 100 mg/L of spectinomycin and cultured for 12 h. Generated *Spc^R* colonies were inoculated on another plate. Each round of inoculation counted for one generation. Strains which generated 100 *Spc^R* colonies among the 100, tested after 20 generations, were considered to be stable. The stable strains were then inoculated into 25-mL LB medium and cultivated at 37 °C. The strains with the highest enzyme activity were selected for the next experiments.

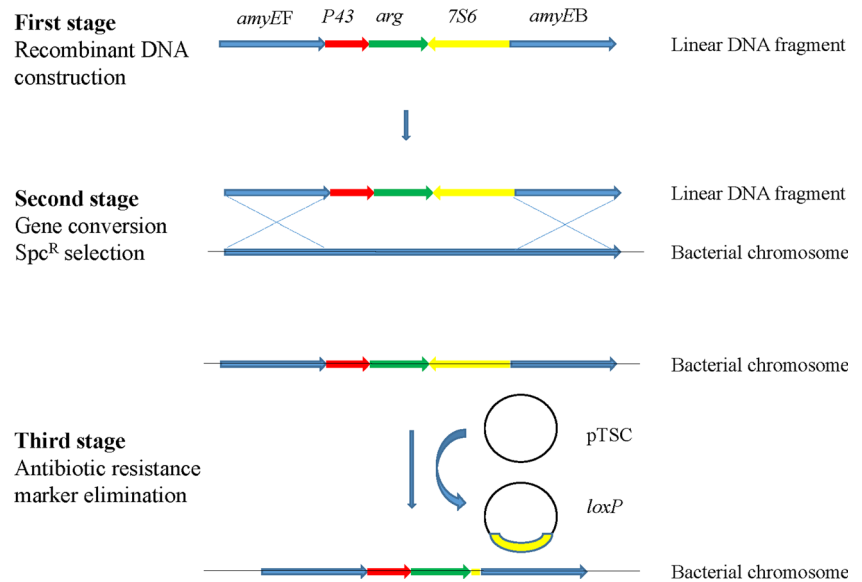
Marker-less recombinant strains

The stability assessment was carried out by transferring single colonies of the marker-less recombinant strains into 25-mL LB medium. After culturing for 24 h at 37 °C, 3% (v/v) cultures were inoculated into another 25-mL fresh LB medium and the enzyme activities were determined. Each round of inoculation counted for one generation and the determinations were performed for a few days.

Further increase of the *arg^{R.pyc}* cassette copy number

The strain that initially possessed 2 *arg^{R.pyc}* cassettes, BSKA-A2 ($\Delta amyE$, $\Delta thrC::arg^{R.pyc}$), was used as the first recipient. Chromosomal DNA of a stable *Spc^R* strain was extracted and used for transformation of BSKA-A2. *B. subtilis* genome with increased copy number was obtained via selection of *Spc^R* transformants. Recombinant strain with *Spc^R* deletion was used as the next recipient, and another stable *Spc^R* strain was considered to be a new donor of chromosomal DNA for copy number increase. In the last round of the *arg^{R.pyc}* cassette amplification, a marker-less strain with multi-copy ectopic insertions of the target gene was obtained.

Fig. 1 Scheme for the *arg^{R.pyc}* cassette integration into designed chromosomal region (insertion at *amyE* site was taken as an example)



The level of arginase expression and the presence of integrated cassettes in the genome were detected by using real-time quantitative PCR (RT-qPCR) and SDS-PAGE analysis.

RT-qPCR analysis of *B. subtilis* integrated cassettes

PCR was prepared based on Bust n' Grab method reported previously (Harju et al. 2004). Genomic DNA was used as the template for the copy numbers of *arg^{R.pyc}* detection with SYBR Green I dye. Primers (*P_{R1}*/*P_{R2}*) were designed from the sequence of *arg^{R.pyc}* and utilized to quantify the *arg^{R.pyc}* copy number. Reaction conditions were established as recommended by SYBR Premix EX *TaqTM* II manual. Each reaction mixture contained 5 μ L of template DNA (1 ng/ μ L). Concentrations of genomic DNA were measured by a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Serial 10-fold dilutions of BSKA-A1 genomic DNA were used to establish the standard curve. To ensure the accuracy, PCR product amplified from BSKA-A1 was sequenced. BSKA was taken as the negative control. In addition, determination of genomic DNA concentration was necessary, as the aim was to obtain a different copy concentration at the same genomic DNA level. All RT-qPCR reactions were performed in triplicate on CFX96 real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Protein analysis

The cells harboring *R. pycnus* arginase were harvested from the culture medium (1 mL) by centrifugation. Harvested cells were resuspended with 200 μ L Tris-HCl buffer (50 mM, pH 7.5). Then, 20 μ L lysozyme (1 mg/mL) was added into the cell suspensions and incubated at 37 $^{\circ}$ C for 1 h and cell

debris was removed by centrifugation. The mixture containing 80 μ L crude enzyme and 20 μ L SDS-PAGE loading buffer (5 \times) was incubated in boiling water for 10 min. Total 5 μ L prepared protein sample was loaded onto each lane of the SDS-PAGE. Purified recombinant *R. pycnus* arginase overexpressed in *E. coli* BL21 (DE3) was taken as a control.

Biosynthesis of L-ornithine with the constructed *B. subtilis*

Biosynthesis of L-ornithine was determined under optimal conditions. A total volume of 100 mL of the conversion mixture consisted of 100 g/L L-arginine, 2 mM Mn^{2+} , and 0.3 g harvested cells. The reaction was performed in a shaking water bath at pH 9.0 and 40 $^{\circ}$ C for 12 h. The concentrations of the product and substrate were analyzed by HPLC at the given time intervals.

Results

Ectopic insertion of *arg^{R.pyc}* cassettes into designed sites

Preciously, a thermostable arginase from *R. pycnus* SK 31.001 has been characterized. The nucleotide sequence of the arginase gene was amplified from the *R. pycnus* chromosome, and primers were designed based on the available gene sequence (GenBank accession number KP702726). DNA amplicons covering a 906-bp open reading frame were sequenced, and the result coincided with the arginase gene. To improve the production level of the *R. pycnus* arginase, a strong promoter *P43* from *B. subtilis* was fused with the arginase gene to generate an *arg^{R.pyc}* expression cassette, which was taken as a monomeric fragment. Furthermore, to eliminate the effect of

the *B. subtilis* arginase, the gene encoding arginase (*argI*) was knocked out.

Several genes have been reported for target gene integration (Härtl et al. 2001), such as *amyE*, *thrC*, *lacA*, *nprB*, and *rib*. In this study, *amyE* and *thrC* were chosen as the target integration sites. Ectopic insertions of *arg^{R.pyc}* were performed to simultaneously inactivate the target integration site genes. The rapid confirmation of a recombinant *B. subtilis* was made by inoculation of cells on LB agar plates containing 0.002% phenol red (shown in Fig. 2). Ammonia, produced by the arginase and the urease reactions from L-arginine, mediated the pH increase. The alkaline drift in pH produced a red coloration around colonies exposed to phenol red. Furthermore, a higher expression level of *arg^{R.pyc}* would lead to a redder and larger coloration region.

Random integration into the bacterial genome

The key step of the random integration strategy was the construction of *arg^{R.pyc}-7S6* sandwiched by “front” and “back” homologous arms which were digested from the recipient chromosome. The strategy of random integration was shown in Fig. 3. During the random integration, linear *Bam*HI-generated fragments consisting of “front” and “back” homologous arms and an *arg^{R.pyc}* cassette were used for double-crossover recombination into the chromosome. These circular fragments which were resistant to *Bam*HI cleavage were integrated into the chromosome via single-crossover recombination.

To select stable recombinant strains, a segregation stability experiment was carried out. Successful random integrations led to form more than 100 *Spc^R* clones; however, only 20 transformants performed 100% segregation stability were used for further experiments.

Further increase of the *arg^{R.pyc}* cassette copy number

The three strains with the highest enzyme activity from the random integrations were selected from the 20 transformants showing 100% segregation stability. These selected strains were designated with labels, such as BSKA-B1 ($1 \times_1, 1 \times_2 \dots 1x_N:: arg^{R.pyc}-7S6$). The number, B1 (B2, B3 for the other strains), indicated the strain number, *N* and *x* indicated the copy numbers and unsure loci in the BSKA chromosome. For the *arg^{R.pyc}* cassette amplification, extracted chromosomal DNA from one *Spc^R* strain was used for transformation of a marker-less strain (Parsons et al. 2014; Würtele et al. 2003). BSKA-A2 ($\Delta amyE, \Delta thrC::arg^{R.pyc}$) was designated as the first recipient, which was initially integrated with two copies of the *arg^{R.pyc}* cassettes. Selection of a *Spc^R* transformant led to a strain with increased copy number. The resistance gene was then eliminated via the *Cre/lox* system. The newly constructed marker-less strain was used as the next recipient, and

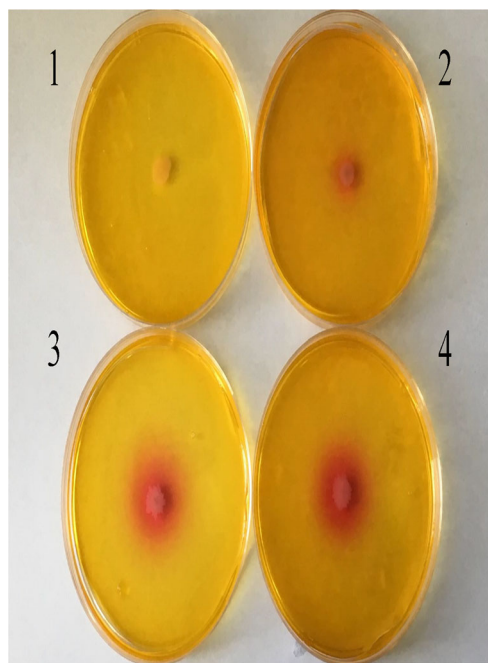


Fig. 2 Inoculation of recombinant strains on LBAP agar: the dependence of arginase production. 1 BSKA, 2 *B. subtilis* 168, 3 BSKA-A1, 4 BSKA-A2

the chromosomal DNA from another *Spc^R* strain was applied in the next transformation. With the step-by-step increase of the integration of the *arg^{R.pyc}* cassette, marker-less strains (BSKA-C1, BSKA-C2, and BSKA-C3) with multi-copy insertions of the *arg^{R.pyc}* cassettes were obtained.

Presence of the integrated cassettes in the genome and the level of arginase expression at each step were determined by RT-qPCR and SDS-PAGE. As shown in Fig. 4a and Fig. 4b, the copy numbers of insertion cassettes in each strain were determined, and BSKA-A1 strain was used as a standard. Three strains with the highest enzyme activity containing two, two, and three copies of the *arg^{R.pyc}* cassettes were constructed via random integration. As mentioned previously, these three strains were segregation stable and could be used as the chromosomal DNA donors for transformation of a marker-less strain. Ultimately, with further increase of copy numbers, three plasmid-less and marker-less strains carrying four, six, and nine copies of the *arg^{R.pyc}* cassettes were obtained. To estimate the level of arginase expression, SDS-PAGE analysis was carried out. As shown in Fig. 4c, the molecular weight of the subunit of *R. pycnus* arginase was determined to be 33 kDa (lane 3). It was reported that the molecular mass of *B. subtilis* arginase was 32 kDa (Yu et al. 2013). Though the *argI* gene encoding *B. subtilis* arginase was knocked out, there was still a light band around 33 kDa (lane 1). There was no arginase activity assayed from BSKA (see below) which revealed that the background band around 33 kDa was not caused by arginase. Compared with this light band, the bands in lanes 4 to 11 were much more obvious and indicated that

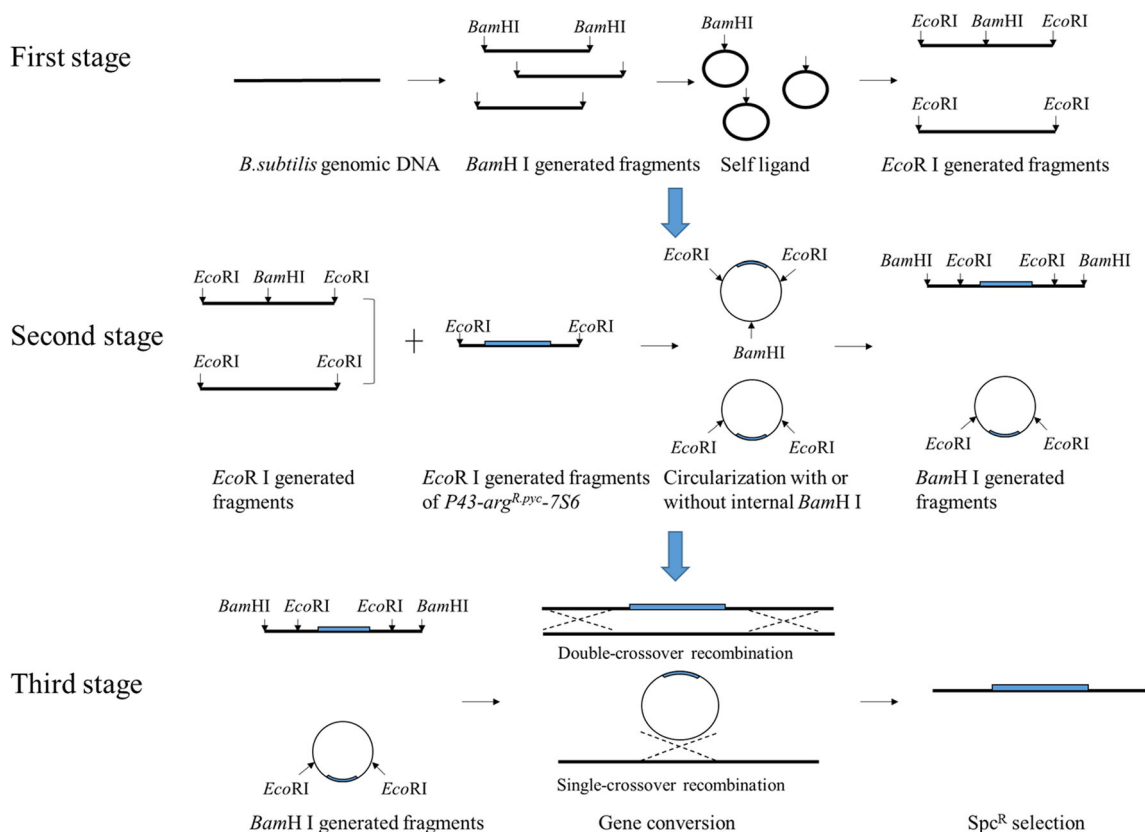


Fig. 3 Scheme for the *arg^{R.pyc}* cassette integration into random chromosomal region. Random integration includes three stages and there would be two kinds of recombination during the gene conversion

the arginase expression level increased with the increase of copy number. The strain with nine copy insertions of the *arg^{R.pyc}* cassettes showed the highest arginase expression level.

Performance of the multi-copy strains

All multi-copy strains were constructed as described above. A set of strains, including BSKA (0 copy), BSKA-A1 (1 copy), BSKA-A2 (2 copies), BSKA-B3 (3 copies), BSKA-C1 (4 copies), BSKA-C2 (6 copies), and BSKA-C3 (9 copies), were used to evaluate the effect of the *arg^{R.pyc}* cassettes on arginase expression in shake flask culture. As shown in Fig. 5a, the growth curves of *B. subtilis* 168 and BSKA-C3 were assayed. Both *B. subtilis* 168 and BSKA-C3 reached the highest OD₆₀₀ value around 14 h. Then, the growth of recombinant strains was measured by determining OD₆₀₀ values after culture for 14 h at 37 °C. The strains with 1–3 copies showed the same growth trend and slightly lower than the wild-type strain (*B. subtilis* 168). However, with copies higher than three, there was a gradual decrease in growth with the increase of *arg^{R.pyc}* cassettes. The total enzyme activity reached a peak value of 14.5 U/mL with strain BSKA-C3, which harbored nine copies of the *arg^{R.pyc}* cassettes (shown in Fig. 5b). Strain BSKA-C3 showed approximately 3-fold and 18-fold higher activity than

the single copy strain BSKA-A1 and *B. subtilis* 168, respectively. Segregation stability of the marker-less strains was determined. As shown in Fig. 5c, the enzyme stability of the recombinant strains was tested for ten generations and the results suggested that stable multi-copy strains were obtained with the insertion of the *arg^{R.pyc}* cassettes into designed and random regions in *B. subtilis* chromosome. Whole-cell conversion was performed under optimal conditions with the nine copy recombinant strain BSKA-C3. As shown in Fig. 5d, the highest conversion rate of L-arginine was reached at 8 h. At this point, 73.8 g/L L-ornithine was obtained with a molar yield of 97.2%. However, there was a slight decrease when reaction time was more than 8 h. This decrease might be a result of consumption of L-ornithine by the cells. Within 8 h, most of the substrate was hydrolyzed with only 2.6 g/L residue.

Discussion

A number of biotransformation process running on a commercial scale revealed that biocatalysis had been a standard technology in the food and chemicals industry. One hundred and thirty-four industrial biotransformation list by Straathof et al. (2002) showed that more than 50 industrial

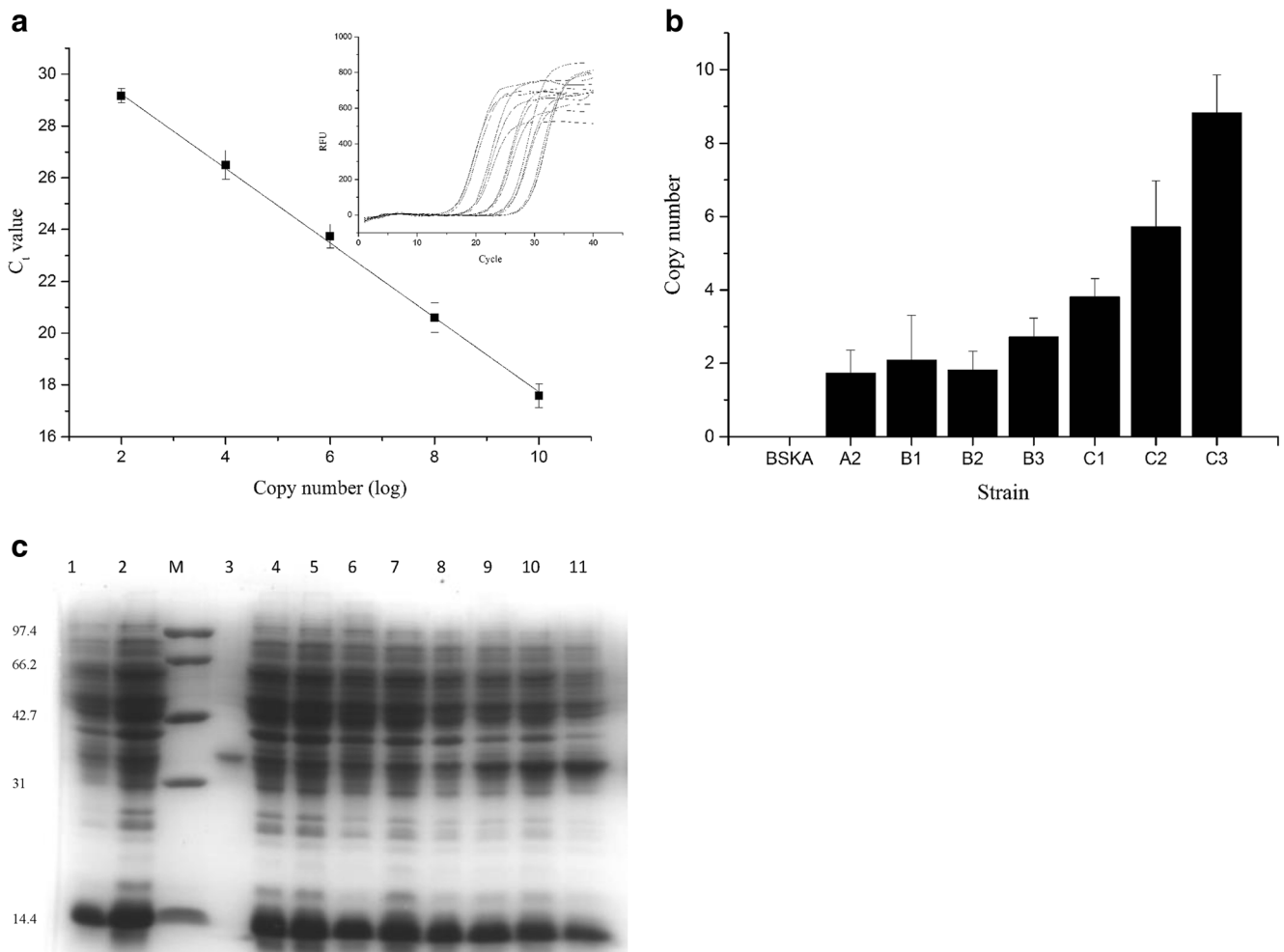


Fig. 4 RT-qPCR analysis of the transformant chromosome and SDS-PAGE of recombinant strains with different *arg^{R.pyc}* copy numbers. **a** Standard curve for *arg^{R.pyc}* DNA concentrations. The standard curve was determined with 10-fold serial dilutions of BSKA-A1 genomic DNA (RT-qPCR results were shown in the small figure). **b** Copy

number determination for each strains. BSKA was taken as a negative control. **c** SDS-PAGE analysis of recombinant strains. Lane M marker, 1 BSKA, 2 *B. subtilis* 168, 3 purified arginase, 4 BSKA-A1, 5 BSKA-A2, 6 BSKA-B1, 7 BSKA-B2, 8 BSKA-B3, 9 BSKA-C1, 10 BSKA-C2, 11 BSKA-C3

biotransformations were used for whole-cell catalysis. *B. subtilis* is an attractive host for the production and secretion of heterologous proteins. We had tried to utilize different Sec-type and Tat-type signal peptides to lead the secretion of *R. pycnus* arginase; however, it has no success yet. It is not clear why the cytoplasmic protein could not be transported with different signal peptides. It was hypothesized that the N terminal regions of this cytoplasmic protein containing order-promoting amino acids might easily form the structure which hindered its secretion (Wang et al. 2013). Therefore, in the present studies, intracellular expression of arginase was chosen.

Arginase from *R. pycnus* SK 31.001 has been characterized and showed that this thermostable enzyme could be applied in L-ornithine biosynthesis (Huang et al. 2016). Introduction of a recombinant plasmid might be a traditional method for expression of arginase. However, structural and segregational instability of plasmids in *B. subtilis* was well-documented

(Shoham and Demain 1991; Fleming and Patching 1994). Compared with the expression with recombinant plasmid, chromosome integration might be a good choice to overcome these disadvantages. To ensure that expression level of the *R. pycnus* arginase, a strong promoter *P43* from *B. subtilis* was fused with the arginase gene to generate an *arg^{R.pyc}* expression cassette. A strategy of multi-copy insertions was used to integrate the *arg^{R.pyc}* cassettes into different designed and random sites in the host chromosome. Southern hybridization might be the most widely used method for copy number determination. However, this method was time-consuming, semi-quantitative, and laborious (Pushnova et al. 2000). Therefore, an RT-qPCR method was established, and the genomic DNA (Zhu et al. 2009) was used directly as the template.

Multi-copy insertions were separated into three parts including designed site insertion, random insertion, and further increase of the *arg^{R.pyc}* cassette copy number. There were two

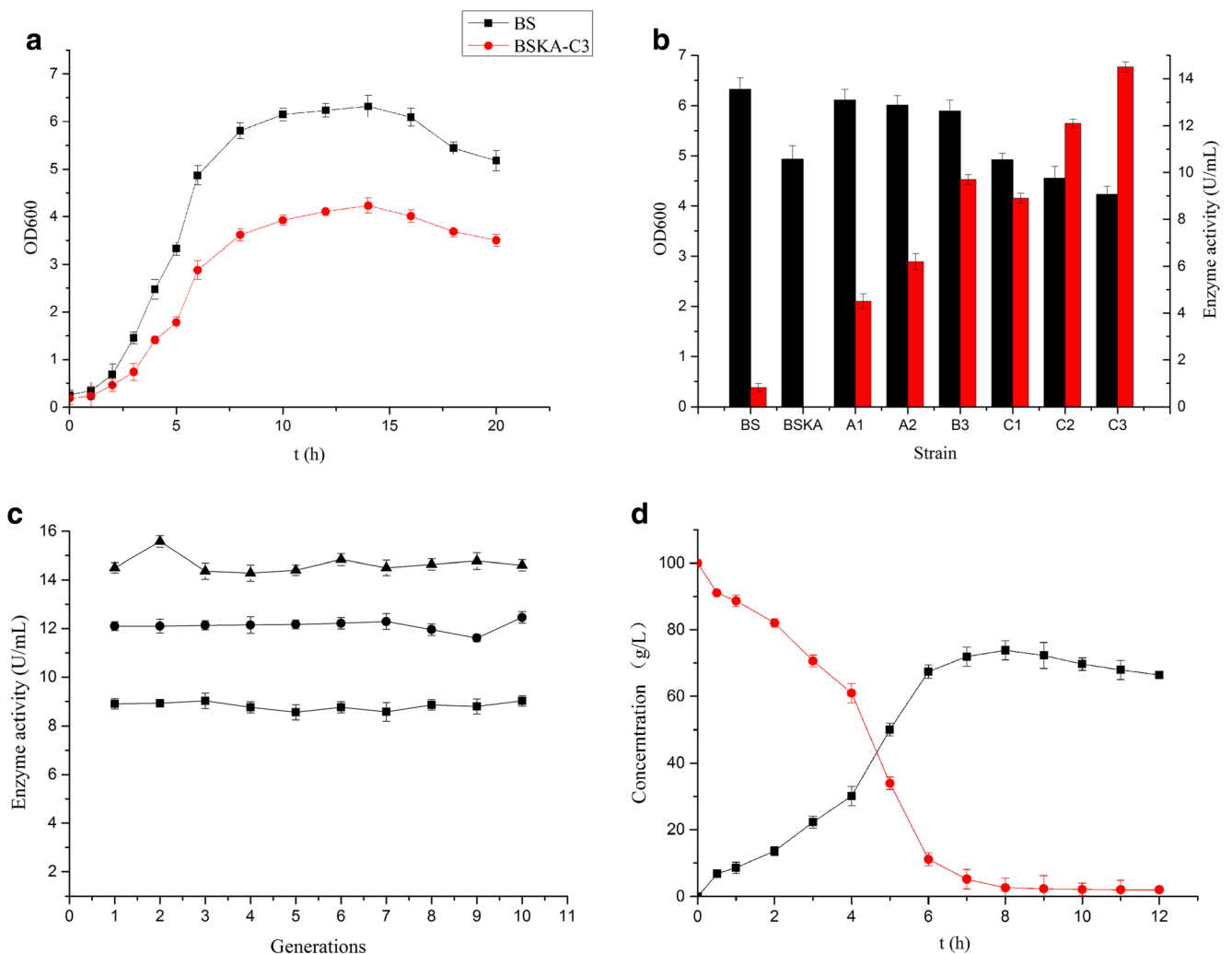


Fig. 5 Performance of the constructed multi-copy integration strains. **a** Growth curve of BS (*B. subtilis* 168) and BSKA-C3. **b** Total enzyme activity (black bar) and growth determination (red bar) of each recombinant strain. Each strain was estimated after cultured at 37 °C for 14 h. **c** Segregation stability of the constructed marker-less strains (9

copies (▲), 6 copies (●), 4 copies (■)). The stability of each strain was estimated by enzyme activity of every generation. **d** Bioconversion for L-ornithine production (L-arginine (●), L-ornithine (■)). The conversion was performed in a shaking water bath at pH 9.5 and 40 °C for 12 h

kinds of homologous recombination during the random insertions. It was supposed that the number of circular-mediated integrations might be more than linear fragment insertions (Schumann 2007), while double-crossover recombination (linear insertions) would be rather stable than single-crossover recombination (circular-mediated integrations) for the recombination-dependent elimination of the cassette in the *B. subtilis* expression system (Schumann 2007). These might explain why only 20 transformants performed 100% segregation stability among the generated more than 200 clones. Random integration of target gene could be easily obtained; however, the copy numbers would be unexpectedly high (most of the integrations were approximately 1–2 copies) (Yomantas et al. 2011). The highest three copies of the *arg^{R.pvc}* cassettes were constructed via random integration. Combination with the designed site insertion and the random

insertion led to a further increase of the *arg^{R.pvc}* cassette copy number. According to the reported studies, there were likely 50–100 copies in a plasmid carrier strain (Wu and Wong 1999). Moreover, 52 copies could be also obtained with repeated cassette insertions into the target chromosome region (Zhu et al. 2009). However, plasmid and repeated cassette insertion might be unstable in *B. subtilis* host (Fleming and Patching 1994). Recombination between repeated cassettes would lead to internal chromosomal deletions through cyclization (He et al. 2016). In some cases, instability might reduce the profitability of fermentations or even make the strain non-viable. Segregation stability was a major factor for the application of recombinant strains that must be taken into consideration (Yomantas et al. 2011). BSKA-C3 with nine copy insertions of *arg^{R.pvc}* cassettes was constructed. Compared with these reported strains, the copy number of chromosomal

integration strain in this study was low. However, segregation stability determination of the marker-less strain showed that BSKA-C3 was relatively stable. Moreover, this constructed plasmid-less strain displayed essentially the same arginase activity (14.5 U/mL) level as the recombinant *B. subtilis* strain carrying expression plasmid with inserted *Bacillus amyloliquefaciens argI* gene (which showed nearly 26-fold higher enzyme activity than the wild-type *B. subtilis* 168 strain) (Wang et al. 2015). The fermentation of recombinant strain was based on the LB medium; then, higher activity could be obtained with the optimization of medium composition. High concentration of residual substrate might make product separation difficult. Whole-cell conversion performed with BSKA-C3 led to a molar yield of 97.2%, which was higher than the reported yield (Song et al. 2014; Wang et al. 2015). In addition, it seemed useful to estimate the random integration sites for the construction of stable recombinant strains. This determination could be carried out by inverse PCR with a further study (Tchetina and Newman 1995).

L-Ornithine could be used as a food additive, so food safety must be taken into consideration by food industry. Previous studies were focused on the bioconversion of L-ornithine production using whole-cell recombinant *E. coli* or the purified arginase (Song et al. 2014; Yu et al. 2013). In contrast to the *E. coli* expression system, *B. subtilis* is a GRAS organism that has been used in the food industry for a long time. In this study, a multi-copy, non-antibiotic resistant *B. subtilis* strain was constructed. This segregation stable recombinant strain showed excellent catalytic activity and would be a good candidate for L-ornithine production in the food industry.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

- Anagnostopoulos C, Spizizen J (1961) Requirements for transformation in *Bacillus subtilis*. *J Bacteriol* 81(5):741–746
- Bron S, Holsappel S, Venema G, Peeters BPH (1991) Plasmid deletion formation between short direct repeats in *Bacillus subtilis* is stimulated by single-stranded rolling-circle replication intermediates. *Mol Genet* 226(1–2):88–96. doi:10.1007/BF00273591
- Fleming GT, Patching DJW (1994) Plasmid instability in an industrial strain of *Bacillus subtilis* grown in chemostat culture. *J Ind Microbiol* 13(2):106–111. doi:10.1007/BF01584107

- Harju S, Fedosyuk H, Peterson KR (2004) Rapid isolation of yeast genomic DNA: bust n' grab. *BMC Biotechnol* 4(1):1–6. doi:10.1186/1472-6750-4-8
- Härtl B, Wehrl W, Wiegert T, Homuth G, Schumann W (2001) Development of a new integration site within the *Bacillus subtilis* chromosome and construction of compatible expression cassettes. *J Bacteriol* 183(8):2696–2699. doi:10.1128/JB.183.8.2696-2699.2001
- He W, Mu W, Jiang B, Yan X, Zhang T (2016) Food-grade expression of d-Psicose 3-epimerase with tandem repeat genes in *Bacillus subtilis*. *J Agr Food Chem* 64(28):5701–5707. doi:10.1021/acs.jafc.6b02209
- Huang K, Zhang T, Jiang B, Mu W, Miao M (2016) Characterization of a thermostable arginase from *Rummeliibacillus pycnus* SK31.001. *J Mol Catal B-Enz*. doi:10.1016/j.molcatb.2016.11.020
- Jalan R, Wright G, Davies NA, Hodges SJ (2007) L-Ornithine phenylacetate (OP): a novel treatment for hyperammonemia and hepatic encephalopathy. *Med Hypotheses* 69(5):1064–1069. doi:10.1016/j.mehy.2006.12.061
- Jeong KJ, Park IY, Kim MS, Kim SC (1998) High-level expression of an endoxylanase gene from *Bacillus* sp. in *Bacillus subtilis* DB104 for the production of xylobiose from xylan. *Appl Microbiol Biotechnol* 50(1):113. doi:10.1007/s002530051264
- Kastner S, Perreten V, Bleuler H, Hugenschmidt G, Lacroix C, Meile L (2006) Antibiotic susceptibility patterns and resistance genes of starter cultures and probiotic bacteria used in food. *Syst Appl Microbiol* 29(2):145–155. doi:10.1016/j.syapm.2005.07.009
- Lee SH, Kim HJ, Shin YA, Kim KH, Lee SJ (2016) Single crossover-mediated markerless genome engineering in *Clostridium acetobutylicum*. *J Microbiol Biotechnol* 26(4):725–729. doi:10.4014/jmb.1512.12012
- Meissner L, Kauffmann K, Wengeler T, Mitsunaga H, Fukusaki E, Büchs J (2015) Influence of nitrogen source and pH value on undesired poly(γ -glutamic acid) formation of a protease producing *Bacillus licheniformis* strain. *J Ind Microbiol Biot* 42(9):1203–1215. doi:10.1007/s10295-015-1640-7
- Parsons JB, Frank MW, Eleveld MJ, Schalkwijk J, Broussard TC, Jonge MID, Rock CO (2014) A thioesterase bypasses the requirement for exogenous fatty acids in the plsX deletion of *Streptococcus pneumoniae*. *Mol Microbiol* 96(1):28–41. doi:10.1111/mmi.12916
- Povelainen M, Miasnikov AN (2007) Production of xylitol by metabolically engineered strains of *Bacillus subtilis*. *J Bacteriol* 128(1):24–31. doi:10.1016/j.jbiotec.2006.09.008
- Pushnova EA, Geier M, Yu SZ (2000) An easy and accurate agarose gel assay for quantitation of bacterial plasmid copy numbers. *Anal Biochem* 284(1):70–76. doi:10.1006/abio.2000.4668
- Sauer C, Syvertsson S, Bohorquez LC, Cruz R, Harwood CR, van Rij T, Hamoen LW (2016) Effect of genome position on heterologous gene expression in *Bacillus subtilis*: an unbiased analysis. *ACS Synth Biol* 5(9):942–947. doi:10.1021/acssynbio.6b00065
- Schumann W (2007) Production of recombinant proteins in *Bacillus subtilis*. *Adv Appl Microbiol* 62(62):137–189. doi:10.1016/S0065-2164(07)62006-1
- Shoham Y, Demain AL (1991) Kinetics of loss of a recombinant plasmid in *Bacillus subtilis*. *Biotechnol Bioeng* 37(10):927–935. doi:10.1002/bit.260371006
- Sietske ADB, Diderichsen B (1991) On the safety of *Bacillus subtilis* and *B. amyloliquefaciens*: a review. *Appl Microbiol Biotechnol* 36(1):1–4. doi:10.1007/BF00164689
- Song W, Niu P, Chen X, Liu L (2014) Enzymatic production of L-ornithine from L-arginine with recombinant thermophilic arginase. *J Mol Catal B-Enz* 110:1–7. doi:10.1016/j.molcatb.2014.09.005
- Straathof AJ, Panke S, Schmid A (2002) The production of fine chemicals by biotransformations. *Curr Opin Biotech* 13(6):548–556. doi:10.1016/S0958-1669(02)00360-9
- Sugino T, Shirai T, Kajimoto Y, Kajimoto O (2008) L-Ornithine supplementation attenuates physical fatigue in healthy volunteers by

- modulating lipid and amino acid metabolism. *Nutr res* 28(11):738–743. doi:10.1016/j.nutres.2008.08.008
- Tchetina E, Newman EB (1995) Identification of Lrp-regulated genes by inverse PCR and sequencing: regulation of two mal operons of *Escherichia coli* by leucine-responsive regulatory protein. *J Bacteriol* 177(10):2679–2683
- Wang G, Chen H, Zhang H, Song Y, Chen W (2013) The secretion of an intrinsically disordered protein with different secretion signals in *Bacillus subtilis*. *Curr Microbiol* 66(6):566–572. doi:10.1007/s00284-013-0315-8
- Wang M, Xu M, Rao Z, Yang T, Zhang X (2015) Construction of a highly efficient *Bacillus subtilis* 168 whole-cell biocatalyst and its application in the production of L-ornithine. *J Ind Microbiol Biotechnol* 42(11):1427–1437. doi:10.1007/s10295-015-1672-z
- Wu SC, Wong SL (1999) Development of improved pUB110-based vectors for expression and secretion studies in *Bacillus subtilis*. *J Biotechnol* 72(1–2):185–195. doi:10.1016/S0168-1656(99)00101-7
- Würtele H, Little KCE, Chartrand P (2003) Illegitimate DNA integration in mammalian cells. *Gene Ther* 10(21):1791–1799. doi:10.1038/sj.gt.3302074
- Yan X, Yu HJ, Hong Q, Li SP (2008) Cre/lox system and PCR-based genome engineering in *Bacillus subtilis*. *Appl Environ Microb* 74(17):5556–5562. doi:10.1128/AEM.01156-08
- Yomantas YA, Abalakina EG, Golubeva LI, Gorbacheva LY, Mashko SV (2011) Overproduction of *Bacillus amyloliquefaciens* extracellular glutamyl-endoropeptidase as a result of ectopic multi-copy insertion of an efficiently-expressed mpr gene into the *Bacillus subtilis* chromosome. *Microb Cell Factories* 10(1):1–10. doi:10.1186/1475-2859-10-64
- Yu JJ, Park KB, Kim SG, Oh SH (2013) Expression, purification, and biochemical properties of arginase from *Bacillus subtilis* 168. *J Microbiol* 51(2):222–228. doi:10.1007/s12275-013-2669-9
- Zhang T, Guo Y, Zhang H, Mu W, Miao M, Jiang B (2013) Arginase from *Bacillus thuringiensis* SK 20.001: purification, characteristics, and implications for L-ornithine biosynthesis. *Process Biochem* 48(4):663–668. doi:10.1016/j.procbio.2013.02.023
- Zhu T, Guo M, Tang Z, Zhang M, Zhuang Y, Chu J, Zhang S (2009) Efficient generation of multi-copy strains for optimizing secretory expression of porcine insulin precursor in yeast *Pichia pastoris*. *J Appl Microbiol* 107(3):954–963. doi:10.1111/j.1365-2672.2009.04279.x