Biotechnol Lett (2015) 37:1243–1248
DOI 10.1007/s10529-015-1783-7

ORIGINAL RESEARCH PAPER

Enhancement of L-valine production in *Bacillus licheniformis* by blocking three branched pathways

Chengwen Liang · Yanli Huo · Gaofu Qi · Xuetuan Wei · Qin Wang · Shouwen Chen

Received: 5 January 2015 / Accepted: 29 January 2015 / Published online: 21 February 2015
© Springer Science+Business Media Dordrecht 2015

Abstract
Objectives *Bacillus licheniformis* WX-02 is used for the production of many valuable chemicals. Here, we have sought to improve L-valine production by blocking the metabolic pathways related to branched-chain amino acids.
Results The synthesis genes of L-leucine (*leuA*) and L-isoleucine (*ilvA*) were deleted to obtain mutant strains. L-Valine yields of WX-02Δ*leuA* and WX-02Δ*ilvA* reached 33.2 and 21.1 mmol/l, respectively, which are 22 and 14 times higher than the wild-type WX-02 (1.53 mmol/l). After further deletion of L-lactate dehydrogenase gene (*ldh*) from WX-02Δ*leuA*, the productivity reached 0.47 mmol/l h, an increase of 19 %.
Conclusion We provide a possibility to over-produce L-valine using genetically-modified *B. licheniformis* using remodeling of the biosynthetic pathway to L-valine.

Keywords *Bacillus licheniformis* · Branched-chain amino acids · Genetically engineered strain · L-Valine · Remodeling metabolic pathway

Introduction

L-Valine (L-Val) is a branched-chain amino acid which is essential for vertebrates and is widely used as a reagent for infusion solutions, cosmetics, foods, and feed industries (Blombach et al. 2008). As an essential amino acid, L-Val is also used for total artificial nutrition and for synthesis of antiviral drugs (Blombach et al. 2007). The fermentative production of L-Val has attracted increasing attentions (Bartek et al. 2008) with a variety of microorganisms being reported as L-Val producers including *Corynebacterium* (Bartek et al. 2010), *Serratia marcescens* (Khachatryan 1986), *Brevibacterium* (Iborra et al. 1992), *Escherichia* (Park et al. 2007) and *Bacillus* (Chattopadhyay and Banerjee 1978).

Biochemically, L-Val is synthesized from two molecules of pyruvate, in a pathway comprising four

Electronic supplementary material The online version of this article (doi:10.1007/s10529-015-1783-7) contains supplementary material, which is available to authorized users.

C. Liang · Q. Wang · S. Chen (✉)
Hubei Collaborative Innovation Center for Green Transformation of Bio-Resources, College of Life Sciences, Hubei University, Wuhan 430062, People's Republic of China
e-mail: chenshouwen@hubu.edu.cn

C. Liang · Y. Huo · G. Qi · X. Wei · S. Chen
State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430070, People's Republic of China

X. Wei
College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, People's Republic of China

reactions, catalyzed by acetohydroxyacid synthase (AHAS/ALS), acetohydroxy acid isomero reductase (AHAIR/ALIR), dihydroxyacid dehydratase (DHAD), and transaminase B. As shown in Fig. 1, two pyruvates are condensed by AHAS/ALS to acetolactate, which is reduced and isomerised to dihydroxyisovalerate (DHIV) by AHAIR/ALIR, and further dehydrated to ketoisovalerate (KIV) catalyzed by DHAD. KIV is finally transformed to L-Val by transaminase B, with glutamate as an amino donor (Bartek et al. 2010). In most organisms, these enzymes also catalyze the biosynthesis of L-isoleucine from pyruvate and α-ketobutyrate. α-Ketobutyrate is synthesized from L-threonine by threonine dehydratase (TD) (Fig. 1). Ketoisovalerate, the last intermediate of L-Val synthesis, also serves as the precursor for L-leucine and pantothenate biosynthesis (Blombach et al. 2007; Bartek et al. 2008).

Bacillus subtilis and *B. licheniformis* can produce L-Val (Chattopadhyay and Banerjee 1978). *B. licheniformis* WX-02 is a previously isolated strain in our laboratory for poly-γ-glutamic acid (γ-PGA) production (Wei et al. 2010), and L-Val was also found in its culture broth (unpublished data). *B. licheniformis* as a L-Val producer has several advantages, such as rapid growth in the media with minimal nutrient requirement, and being classified as "generally recognized as safe". *B. licheniformis* is also a thermophile which can grow above 40 °C thereby reducing the risk of bacterial contamination. However, the wild type of *B. licheniformis* WX-02 only produces a moderate amount of L-Val.

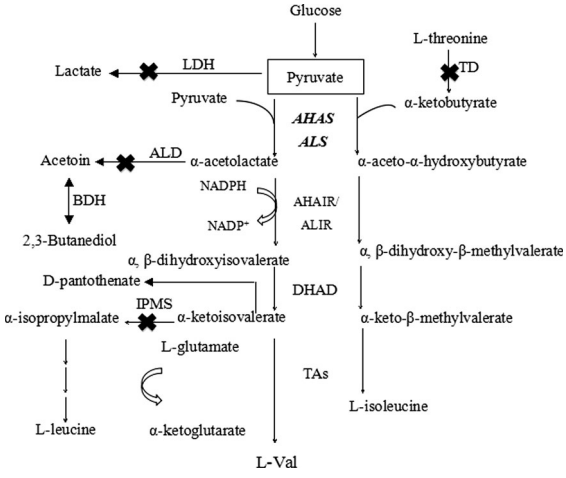

Fig. 1 Biosynthesis pathway of L-Val in *B. licheniformis*. *AHAS/ALS* acetohydroxyacid synthase, *AHAIR/ALIR* acetohydroxy acid isomero reductase, *DHAD* dihydroxyacid dehydratase, *TAs* transaminases, *LDH* lactate dehydrogenase, *ALD* acetolactate decarboxylase, *IPMS* isopropylmalate synthase. *The filled forks* mean the successfully deleted genes in this study

In *B. licheniformis*, biosynthesis of the other branched-chain amino acids and other metabolic pathways consumes the substrate, energy, coenzymes and NADH/NADPH, which then decreases the production of L-Val. Therefore, remodeling the metabolic pathway is needed for enhanced production of L-Val. The aim of this work is to redirect the metabolic fluxes towards L-Val by blocking the other branched-chain amino acid biosynthesis and overflow metabolic pathways in *B. licheniformis* WX-02.

Materials and methods

Cell strains, plasmids, primers and growth media

Experiments were performed with the strains and plasmids listed in Tables 1 and 2. The oligonucleotide primers, listed in Supplementary Table 1, were designed based on the *B. licheniformis* WX-02 genome sequence (GenBank accession no. AHIF00000000) (Yangtse et al. 2012). LB medium was used for culture of *Escherichia coli* DH5α and *B. licheniformis*. Medium used for the fermentation of L-Val (Radmacher et al. 2002) was prepared by previously described methods with minor modifications, consisting of (per liter): 100 g glucose, 2 g corn steep liquor, 15 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 3 g $CaCO_3$.

Chemicals and materials for cloning

All other chemicals were of analytical grade supplied by Sinopharm Chemical Reagent (China). T4 DNA ligase, DNA marker and TransStart Fast *Pfu* DNA Polymerase were purchased from Takara Bio (China) or TransGen Biotech (China). Nucleotide sequences were determined by Beijing Genomics institution (China).

Construction of plasmids

Bacillus licheniformis WX-02 was cultured in LB medium overnight, then collected for extraction of

Table 1 Plasmids used in this study

Plasmids	Characteristics	Source
T2(2)-ori	*E. coli* and *B. subtillis* shuttle vector, ori_{ts}, Kan^r	Stored in this lab
T2(2)-oriΔ*ilvA*	T2(2)-ori derivative harboring homologous arms for *ilvA* knock-out, ori_{ts}, Kan^r	This study
T2(2)-oriΔ*leuA*	T2(2)-ori derivative harboring homologous arms for *leuA* knock-out, ori_{ts}, Kan^r	This study
T2(2)-oriΔ*ldh*	T2(2)-ori derivative harboring homologous arms for *ldh* knock-out, ori_{ts}, Kan^r	Stored in this lab

ori_{ts} temperature-sensitive origin of replication, Kan^r kanamycin resistance

Table 2 Strains used in this study

Strains	Characteristics	Source
Escherichia coli		
DH5α	F^- Φ80d/*lac*ZΔM15, Δ(*lacZYA-argF*) U169, *recA*1, *endA*1, *hsdR*17 (r_K^-, m_K^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1	Stored in this lab
Bacillus licheniformis		
WX-02	CCTCC M208065, wild type	Stored in this lab
WX-02Δ*ilvA*	Wild type with *ilvA* deleted	This study
WX-02Δ*leuA*	Wild type with *leuA* deleted	This study
WX-02Δ*leuA*Δ*ldh*	Wild type with *leuA* and *ldh* deleted d	This study

Kan^r kanamycin resistance

genomic DNA (Sohail 1998). The gene, *ilvA,* encoding TD was deleted by the double-crossover homologous recombination method (Qi et al. 2014). First, two approximately 500 bp homologous arms of the 5′- and 3′-coding regions of *ilvA* gene were amplified by PCR from the genomic DNA of *B. licheniformis* WX-02 with the primers *ilvA*KF1 and *ilvA*KR1, *ilvA*KF2 and *ilvA*KR2, respectively (Supplementary Table 1). These two homologous arms were ligated by overlapping extension PCR (SOE-PCR) with the primers *ilvA*KF1 and *ilvA*KR2 (Supplementary Table 1) (Wang et al. 2012; Qi et al. 2014). The DNA fragment was subcloned into vector T2(2)-ori (Qi et al. 2014) joined by *Bam*HI and *Xba*I restriction sites. The resulting plasmid was further verified by sequencing. A recombinant vector for *ilvA* knock-out was designated as T2(2)-oriΔ*ilvA*. The other two T2(2)-ori-based plasmids including T2(2)-oriΔ*leuA* and T2(2)-oriΔ*ldh* were also constructed with the appropriate primers (Supplementary Table 1) by the above methods for deletion of *leuA* encoding for α-isopropylmalate synthase and *ldh* encoding for lactate dehydrogenase in *B. licheniformis* WX-02.

Construction of *ilvA* and *leuA* knock-out strains of WX-02

Competent cells of *E. coli* DH5α and *B. licheniformis* WX-02 were prepared for transformation of constructed plasmids using standard protocols (see also Xue et al. 1999). *E. coli* DH5α was transferred with T2(2)-oriΔ*ilvA* or T2(2)-oriΔ*leuA* plasmid and then cultured in LB medium with kanamycin (20 μg/ml). The plasmid T2(2)-oriΔ*ilvA* or T2(2)-oriΔ*leuA* isolated from the recombinant *E. coli* was used for transforming *B. licheniformis* WX-02.

The knock-out of *leuA* or *ilvA* gene in *B. licheniformis* WX-02 was done by our previously established methods (Qi et al. 2014). Briefly, WX-02 was electrotransformed with T2(2)-oriΔ*ilvA* or T2(2)-oriΔ*leuA*, the transformants were then selected by kanamycin resistance (20 μg/ml) followed by verification with PCR using the primers *ilvA*KF1 and *ilvA*KR2 for WX-02Δ*ilvA*, and *leuA*KF1 and *leuA*KR2 for WX-02Δ*leuA* (Supplementary Table 1), respectively. The selected positive transformants were cultured in LB medium containing kanamycin

(20 μg/ml) at 45 °C for 8 h to promote the first crossover in the cells.

The successful first crossover was analyzed by PCR. Briefly, the cells with kanamycin resistance were further selected by PCR with primers of Δ*ilvA* A single crossover-F and Δ*ilvA* A single crossover-R for crossover at upstream of *ilvA*, and with primers of Δ*ilvA* B single crossover-F and Δ*ilvA* B single crossover-R for crossover at downstream of *ilvA*, respectively (Supplementary Table 1). The single crossover of *leuA* in WX-02 was also verified by PCR with the appropriate primers (Supplementary Table 1).

The selected colonies with single crossover were repeatedly cultured in LB medium at 37 °C for 8 h. After serial transfer without antibiotics, cells were plated on LB agar plates for selection of kanamycin-sensitive colonies. The *ilvA* or *leuA* knock-out strain was selected by the second crossover, and confirmed by PCR with primers of *ilvA*YF and *ilvA*YR for WX-02Δ*ilvA*, and with *leuA*YF and *leuA*YR for WX-02Δ*leuA*, respectively (Supplementary Table 1).

Construction of WX-02Δ*leuA*Δ*ldh*

The mutant strain of WX-02Δ*leuA*Δ*ldh* was constructed based on the parental strain of WX-02Δ*leuA*. T2(2)-oriΔ*ldh* was used for transforming WX-02Δ*leuA*, then the transformants were selected and verified for the successful single crossover also the double crossover using PCR with the appropriate primers (Supplementary Table 1) by the methods described above, followed with verification by nucleotide sequencing.

Production of L-Val by mutant strains

Single colonies of the wild strain WX-02, and the mutant strains of WX-02Δ*leuA*, WX-02Δ*ilvA* and WX-02Δ*leuA*Δ*ldh*, were transferred into 250 ml flasks containing 50 ml LB medium. The flasks were incubated at 37 °C and 180 rpm for 8 h until the OD_{600} of culture reached 4.2. The cells were then subcultured for 84 or 96 h at the same conditions for producing L-Val. The samples were collected periodically to determine the time course of cell density, residual glucose and L-Val concentrations.

Analysis

Cell densities were determined from the OD_{600} values. The concentration of residual glucose was measured by a biosensor equipped with a glucose oxidase electrode. L-Val was determined by GC after derivatization with ethyl chlorocarbonate (Mudiam and Ratnasekhar 2013). The production of acetoin, 2,3-butanediol and lactate in the broth were analyzed, respectively, by GC (Qi et al. 2014) and HPLC (Santos et al. 2014).

Results

Establishment of WX-02Δ*ilvA* and WX-02Δ*leuA* knock-out strains

The T2(2)-ori plasmid was used for deletion of *ilvA* and *leuA* genes. The PCR product amplified from the genomic DNA of *ilvA* knock-out strain was about 1,498 bp, while a DNA fragment about 2,767 bp containing the *ilvA* gene was amplified from the genomic DNA of WX-02 using primers of *ilvA*YF and *ilvA*YR (Supplementary Table 1). The PCR product from *ilvA* knock-out strain was purified and sequenced. No other mutation than the deletion was found (data not shown), suggesting the successful construction of the *ilvA* deficient strain WX-02 Δ*ilvA*.

Similarly, the PCR product amplified from the genomic DNA of *leuA* knock-out strain was about 1,224 bp, while a DNA fragment about 2,781 bp containing the *leuA* gene was amplified from the genomic DNA of WX-02 by using primers *leuA*YF and *leuA*YR (Supplementary Table 1), suggesting the successful construction of the *leuA* deficient strain WX-02 Δ*leuA*.

L-Val production by WX-02Δ*ilvA* and WX-02Δ*leuA*

L-Val production by the mutant strains is shown in Fig. 2. As expected, the L-Val production of both WX-02Δ*ilvA* and WX-02Δ*leuA* were higher than that of WX-02. WX-02 produced the maximum 1.53 mmol/l L-Val production, while the L-Val produced by the mutant strains of WX-02Δ*ilvA* and WX-02Δ*leuA* reached a maximum 21 mmol/l (13.8 times of WX-

02) at 72 h and 33.2 mmol/l (21.7 times of WX-02) at 84 h, respectively. These data confirm the deletion both of *leuA* and *ilvA* gene significantly increases L-Val production, and the deletion of *leuA* showed a higher L-Val production than the deletion of *ilvA*.

Establishment of WX-02Δ*leuA*Δ*ldh* based on WX-02Δ*leuA*

The T2(2)-ori plasmid was used for deletion of *ldh* gene. The PCR product amplified from WX-02Δ*leuA*Δ*ldh* was about 1,291 bp, while a DNA fragment about 2,251 bp containing the *ldh* gene was amplified from the genomic DNA of WX-02Δ*leuA* using primers of Δ*ldh*-F and Δ*ldh*-R (Supplementary Table 1) as negative control. The PCR products were purified and sequenced, and no other mutation than the deletion was found, suggesting the successful construction of the deficient strains of WX-02Δ*leuA*Δ*ldh*.

L-Val production by WX-02Δ*leuA*Δ*ldh*

The production of lactate was also detected during fermentation. The results showed no L-lactate was detectable after the *ldh* gene was deleted from WX-02Δ*leuA*. Figure 3a shows the biomass of WX-02Δ*leuA*Δ*ldh* is similar to that of WX-02Δ*leuA*. The trend of glucose consumption of WX-02Δ*leuA*Δ*ldh* is similar to that of WX-02Δ*leuA*.

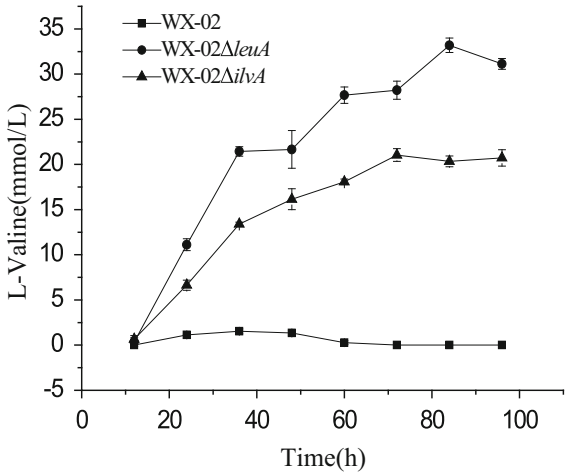

Fig. 2 Comparison of L-Val production by wild type WX-02, WX-02Δ*ilvA*, WX-02Δ*leuA*

Figure 3b shows L-Val production by the mutant strains. L-Val production was similar in WX-02Δ*leuA*Δ*ldh* and WX-02Δ*leuA* in the first stage of fermentation (12–24 h). After 24 h, WX-02Δ*leuA*Δ*ldh* produced more L-Val than the parental strain WX-02Δ*leuA*. At 36, 48 and 72 h, the L-Val production of WX-02Δ*leuA*Δ*ldh* increased by 23.4, 20.2 and 18.9 % respectively, compared to that of WX-02Δ*leuA*. After 72 h, the L-Val production of WX-02Δ*leuA*Δ*ldh* reached the maximum 33.6 mmol/l, higher than that of WX-02Δ*leuA* (27.2 mmol/l). The fermentation time for the maximum L-Val production was 72 h for WX-02Δ*leuA*Δ*ldh*, shorter than that for WX-02Δ*leuA*

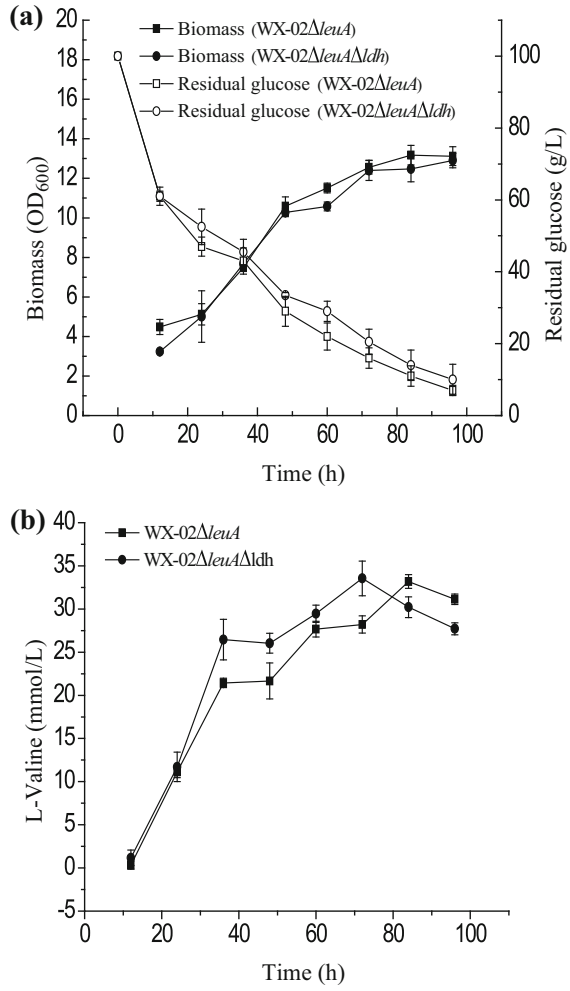

Fig. 3 Comparison of L-Val production, cell growth, and glucose consumption profile by WX-02Δ*leuA* and WX-02Δ*leuA*Δ*ldh*. **a** Cell density and residual glucose concentration. **b** L-Val production. Data were expressed as mean ± standard errors (SE) of three replicates

(84 h). The productivity of WX-02Δ*leuA*Δ*ldh* reached 0.47 mmol/l h, 19 % higher than that of WX-02Δ*leuA*. These data indicated the further deletion of *ldh* gene improved L-Val productivity.

Discussion

Bacillus licheniformis contains a strong metabolic flux from pyruvate to branched-chain amino acids such as L-Val (data not shown), leading us to explore the potential of producing L-Val using this strain. Based on the strong ability of *B. licheniformis* to convert glucose to various branched-chain amino acids, we modified the metabolic flux towards L-Val synthesis. The syntheses of L-leucine and L-isoleucine complete the precursors, energy, coenzymes and NADH/NADPH with that of L-Val (Blombach et al. 2007; Bartek et al. 2010). Therefore, the inhibition of L-leucine and L-isoleucine syntheses is beneficial for L-Val synthesis by redirecting the metabolic fluxes towards L-Val (Blombach et al. 2007, 2008). As expected, the L-Val synthesis significantly enhanced after the deletion of *leuA* or *ilvA* gene in *B. licheniformis* WX-02.

In addition to branched-chain amino acids, a strong overflow metabolic pathway from pyruvate to lactate was also found in *B. licheniformis*. We further deleted the key enzyme gene, *ldh,* for lactate formation, which further increased L-Val productivity. Pyruvate is converted to acetoin and 2,3-butanediol by acetolactate decarboxylase encoded by gene *alsD*, but the deletion of *alsD* did not improve L-Val production (data not shown here). Collectively, our studies provided a possibility to highly produce L-Val by remodeling metabolic pathway for L-Val biosynthesis.

Acknowledgments This work was supported by National Program on Key Basic Research Project (973 Program, No. 2015CB150505).

Supporting Information Supplementary Table 1 – Primers used for PCR in this study.

References

Bartek T, Makus P, Klein B, Lang S, Oldiges M (2008) Influence of L-isoleucine and pantothenate auxotrophy for L-valine formation in *Corynebacterium glutamicum* revisited by metabolome analyses. Bioprocess Biosyst Eng 31:217–225

Bartek T, Blombach B, Zönnchen E, Makus P, Lang S, Eikmanns BJ, Oldiges M (2010) Importance of NADPH supply for improved L-valine formation in *Corynebacterium glutamicum*. Biotechnol Prog 26:361–371

Blombach B, Schreiner ME, Holatko J (2007) L-Valine production with pyruvate dehydrogenase complex-deficient *Corynebacterium glutamicum*. Appl Environ Microbiol 73:2079–2084

Blombach B, Schreiner ME, Bartek T, Oldiges M, Eikmanns BJ (2008) *Corynebacterium glutamicum* tailored for high-yield L-valine production. Appl Microbiol Biotechnol 79:471–479

Chattopadhyay SP, Banerjee AK (1978) Production of valine by a *Bacillus* sp. Z Allg Mikrobiol 18:243–254

Iborra JL, Obon JM, Manjon A, Canovas M (1992) Analysis of a laminated enzyme membrane reactor for continuous resolution of amino acids. Biotechnol Appl Biochem 15:22–30

Khachatryan AG (1986) Dependence of valine production by *serratia marcescens* on the ion composition of the fermentation. Microbiology 22:554–556

Mudiam MK, Ratnasekhar Ch (2013) Ultra sound assisted one step rapid derivatization and dispersive liquid–liquid microextraction followed by gas chromatography–mass spectrometric determination of amino acids in complex matrices. J Chromatogr A 1291:10–18

Park JH, Lee KH, Kim TY, Lee SY (2007) Metabolic engineering of *Escherichia coli* for the production of L-valine based on transcriptome analysis and in silico gene knockout simulation. Proc Natl Acad Sci 104:7797–7802

Qi G, Kang Y, Li L, Xiao A, Zhang S, Wen Z, Xu D, Chen S (2014) Deletion of *meso*-2,3-butanediol dehydrogenase gene *budC* for enhanced D-2,3-butanediol production in *Bacillus licheniformis*. Biotechnol Biofuels 7(1):16

Radmacher E, Vaitsikova A, Burger U, Krumbach K, Sahm H, Eggeling L (2002) Linking central metabolism with increased pathway flux: L-valine accumulation by *Corynebacterium glutamicum*. Appl Environ Microbiol 68(5):2246–2250

Santos CC, Libeck BD, Schwan RF (2014) Co-culture fermentation of peanut-soy milk for the development of a novel functional beverage. Int J Food Microbiol 186(1):32–41

Sohail M (1998) A simple and rapid method for preparing genomic DNA from gram-positive bacteria. Mol Biotechnol 10(2):191–193

Wang Q, Chen T, Zhao X, Chamu J (2012) Metabolic engineering of thermophilic *Bacillus licheniformis* for chiral pure D-2,3-butanediol production. Biotechnol Bioeng 109:1610–1621

Wei X, Ji Z, Chen S (2010) Isolation of halotolerant *Bacillus licheniformis* WX-02 and regulatory effects of sodium chloride on yield and molecular sizes of poly-γ-glutamic acid. Appl Biochem Biotechnol 160(5):1332–1340

Xue G, Johnson JS, Dalrymple BP (1999) High osmolarity improves the electro-transformation efficiency of the gram-positive bacteria *Bacillus subtilis* and *Bacillus licheniformis*. J Microbiol Methods 34(3):183–191

Yangtse W, Zhou Y, Lei Y, Qiu Y, Wei X, Ji Z, Qi G, Yong Y, Chen L, Chen S (2012) Genome sequence of *Bacillus licheniformis* WX-02. J Bacteriol 194(13):3561–3562