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Screening, Characterization and Utilization of D-amino Acid Aminotransferase to Obtain D-phenylalanine[1]

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Abstract—D-phenylalanine (D-Phe) is an important side-chain building block for semi-synthetic penicillins and cephalosporins. To synthesize D-Phe, D-amino acid aminotransferases (Dat) from *Bacillus subtilis* WB600, *Bacillus licheniformis* ATCC 14580, and *Bacillus amyloliquefaciens* were firstly compared. The theoretical 3 dimensional models of Dat were constructed, which showed appropriate configuration for synthesis of D-Phe in virtual screening. The comparison 3 different Dat on D-Phe formation was tested. All the heterogenous Dat showed high thermostability and pH stability, while Dat from *B. subtilis* demonstrated a better potential for Phe production than those from *B. licheniformis* and *B. amyloliquefaciens*. Then the *dat* from *B. subtilis* was expressed in an L-Phe producing chassis of *Escherichia coli* W14 (pR15ABK) to obtain D-Phe and different factors affecting D-Phe accumulation were tested. The engineered strain E. coli BCEA (pR15ABKApRdatBS) accumulated 1.72 g/L D-Phe in a 15-L jar fermenter which is the highest fermentation concentration that had been reported.

Keywords: D-amino acid aminotransferases, 3 dimensional models, configuration for synthesis of D-Phe, D-phenylalanine, semi-synthetic penicillins

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D-phenylalanine (D-Phe) is an important intermediate of semi-synthetic antibiotics such as ampicillin [1], penicillin and cephalosporin [2] and required for the preparation of bacitracin and fungisporin [3]. D-Phe is presently produced by chemical resolution of a racemic mixture [4] derived from petrochemical feedstock. In contrast to present method, the chemicals (such as L-Tyr and L-Thr) produced by microorganisms are usually chiral pure compounds and the sources are renewable without utilization of environmentally unfriendly organic solvents. The fermentative production of chemicals based on green chemistry and renewable resources makes microbial production of D-Phe of great interest.

Microbial production of aromatic amino acids like L-Phe, and L-Thr has been achieved in the past decades [5–7]. Although free D-Phe has not been testified in the biosphere, modifying the L-Phe synthetic pathway of *Escherichia coli* could construct a D-Phe synthetic pathway [8]. The prerequisite for D-Phe biosynthesis is the isolation of an effective D-Phe aminotransferase that converts phenylpyruvate (the precursor of L-Phe) to D-Phe. Although a specific D-Phe aminotransferase has not been reported, the D-amino acid aminotransferase (Dat, EC: 2.6.1.21) in *Bacillus* species [9, 10] can be used to prepare a broad range of D-amino acids due to its broad substrate specificity. To increase the D-Phe yield, the L-amino acid aminotransferase is also an important factor to decrease byproduct formation. Besides, the supplement of amino donor, D-Ala, needs to be discussed to synthesize D-Phe from phenylpyruvate.

In this paper, the Dat from different *Bacillus* strains were analyzed based on their 3D models by using the homology model and the virtual screening. Then the Dat from *Bacillus subtilis, Bacillus licheniformis* and *Bacillus amyloliquefaciens* were firstly amplified, expressed in *E. coli*, purified, and characterized. Factors affecting D-Phe accumulation were tested. Finally, a D-Phe synthetic operon was constructed to biosynthesize D-Phe in a 15-L jar fermenter.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids were listed in Table 1. The primers were given in Table 2. All bacteria were cultured at 37°C in Luria Bertani (LB) medium during strain construction, except the strains containing promoter p_R or p_L (growth at 33°C). Antibiotics (ampicillin 100 μg/L, kanamycin 30 μg/L, or chloroamphenicol 25 μg/L) were added to maintain the plasmids. Gene deletion strains derived from E. coli W14 were constructed as previously reported [11].

[1] The article is published in the original.

Table 1. Microbial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	Source or reference
Strains		
E. coli JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi (Lac-proAB) proAB$^+$ lacI^q lac ZM15*	Stratagene, USA
B. subtilis WB600	Wild-type	CICIM-CU
B. licheniformis ATCC 14580	Wild-type	CICIM-CU
B. amyloliquefaciens	Wild-type	CICIM-CU
E. coli BL21(DE3)	*F$^-$ ompThsdSB (r_B^- m_B^-) gal dcm (DE3)*	Novagen, USA
E. coli W14	Tyrosine auxotroph, *Δcrr*	[7]
BC	The same as W14 *ΔtyrB ΔaspC*	[7]
BCE	The same as W14 *ΔtyrB ΔaspC ΔilvE*	This work
BCA	The same as W14 *ΔtyrB ΔaspC ΔdadA*	This work
BCEA	The same as W14 *ΔtyrB AaspC ΔilvE ΔdadA*	This work
Plasmids		
pMD18-T Vector	*amp^r*	TaKaRa, Japan
pET-28a(+)	*kan^r lacI*	Novagen, USA
pR15ABK	*kan^r aroG15 pheA^{fbr} aroK ydiB* λcIst857	[7]
pETdat$_{BS}$	*kan^r dat lacI dat$_{BS}$*	This work
pETdat$_{BL}$	*kan^r dat lacI dat$_{BL}$*	This work
pETdat$_{BA}$	*kan^r dat lacI dat$_{BA}$*	This work
pR15ABKpRdat$_{BS}$	*kan^r aroG15 pheA^{fbr} aroK ydiB λcI^{ts}857 pR dat$_{BS}$*	This work
pR15ABKpRdat$_{BL}$	*kan^r aroG15 pheA^{fbr} aroK ydiB λcI^{ts}857 pR dat$_{BL}$*	This work
pR15ABKpRdat$_{BA}$	*kan^r aroG15 pheA^{fbr} aroK ydiB λcI^{ts}857 pR dat$_{BA}$*	This work
pR15ABKApRdat$_{BS}$	*kan^r aroG15 pheA^{fbr} aroK ydiB λcI^{ts}857 pR dat$_{BS}$ alr*	This work
pR15ABKApRdat$_{BL}$	*kan^r aroG15 pheA^{fbr} roK ydiB λcI^{ts}857 pR dat$_{BL}$ alr*	This work
pR15ABKApRdat$_{BA}$	*kan^r aroG15 pheA^{fbr} roK ydiB λcI^{ts}857 pR dat$_{BA}$ alr*	This work

Structural-based screening of Dat from *Bacillus* species. The theoretical 3D models of Dat were constructed by Swiss Model server [12, 13] by homology modeling. The molecular configuration of D-Phe was downloaded from http://zinc.docking.org/. The virtual screening was carried out using AutoDockTools-1.5.6 software to predict whether D-Phe be the product of enzyme reaction. AutoGrid version 4.0 was used to create affinity grids centered on the active site. AutoDock version 4.0 was used to simulate ligand-receptor docking. For each Dat, 100 hits were examined; the configurations with the D-Phe binding at the expected active site were analyzed to predict the capability to produce D-Phe.

Amplification and expression of *dat* genes. Three *dat* genes from *B. subtilis* WB600, *B. licheniformis* ATCC 14580 and *B. amyloliquefaciens*, described as *d*at$_{BS}$, *d*at$_{BL}$, and *d*at$_{BA}$, were amplified from respective chromosomal DNA, then ligated with pET-28a(+) and transferred into E. coli BL21(DE3) [14]. E. coli BL21(DE3) with the target plasmid was incubated at 37°C for 12 h and used to inoculate 2.5 mL in 50 mL TB medium [15]. At OD_{600} of 1.0, the T_7 promoter was induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and cultured at 25°C for 16 h. The cells were harvested by centrifugation at 12000 g for 5 min, washed and resuspended with 100 mM Na-phosphate buffer (pH 7.5) and broken by sonication (30 × 1 s burst with 10 s rests). As a control, *E. coli* BL21(DE3)

Table 2. The sequences of the oligonucleotide primers used in the study

Primer name	Sequence (5' → 3') Homology extensions or restriction sites are underlined	Restriction sites
dadA-inactivati on_FW	GGTTCGTCTCGACGGTACGCAGTTCCAG TTGAAATGGATGTGGCAAATGTTACGTA ACTGCGACACCAGCGTAGGCTGGAGCT GCTTCG	
dadA-inactivati on_RV	CGAACCACCATCTCCAGCGTTTCACGAC GCGGTTGCAACAGCTCGGTATTAAAACC AACAATCTCCGCCATTCCTCCGGGGATC CGTCG	
dadA1	GAGTCAGGGAGATGTGAG	
dadA2	CTCCAGATCCTGAGCATG	
K1	AGGCTATTCGGCTATGACTG	
K2	GGACAGGTCGGTCTTGACAA	
BS-datjpET-Ba mHI_FW	CGCGGATCCGATGATGATGATGATAAAA TGAAGGTTTTAGTCAATGGCC	*Bam*HI
BS-dat-XbaI-RV	CTAGTCTAGACTTTATGAAATGCTAGCA GCCTGTTG	*Xba*I
BL-dat_pET-BamHI_FW	CGCGGATCCGATGATGATGATGATAAAA TGAAAGTTCTTTTTAACGG	*Bam*HI
BL-dat-XbaI-RV	CTAGTCTAGACTTTAAACCGTTTTGGCT GTTTCCGAC	*Xba*I
BA-dat_pET-NheI_FW	CTACTAGCTAGCGATGATGATGATGATAA AATGAAGGCATTAGTAAACG	*Nhe*I
BA-dat-HindIII RV	GGCCCAAGCTTATTCCGGCCTTTTTTCC GCTTATGA	*Hin*dII
BS-dat-SmaI-SD-FW	CTCCCCCGGGAAGGAGGAACAGACATG AAGGTTTTAGTCAATGG	*Sma*I
BS-dat-XbaI-AflII-RV	CATGCTTAAGCATGAACCGTGCTCTAGA GCATTATGAAATGCTAGCAGCC	*Xba*I
BL-dat-SmaI-SD-FW	CTCCCCCGGGAAGGAGGAACAGACATG AAAGTTCTTTTTAACGG	*Sma*I
BL-dat-XbaI-AflII-RV	CATGCTTAAGCATGAACCGTGCTCTAGA GCATTAAACCGTTTTGGCTGT	*Xba*I
BA-dat-SmaI-SD-FW	CTCCCCCGGGAAGGAGGAACAGACATG AAGGCATTAGTAAACGG	*Sma*I
BA-dat-XbaI-AflII-RV	CATGCTTAAGCATGAACCGTGCTCTAGA GCATTATGATTGAACTCCGATCTCC	*Xba*I
BglII-SmaI-Ter manitor-pR-FW	GGAAGATCTTCCTCCCCCGGGGGAAAG CATAGGGTTTGCAGAATCCCTGCTTATAT CTAACACCGTGCGTGT	*Sma*I
BglII-EcoRV-Termanitor-pR-FW	GGAAGATCTTCCAAAGATATCAAGCATA GGGTTTGCAGAATCCCTGCTTATATCTAA CACCGTGCGTGT	*Bgl*II
alr-XbaI-SD-FW	GCTCTAGAGCAAGGAGGAACAGACATG CAAGCGGCAACTGTTG	*Xba*I
alr-AflII-RV	CATGCTTAAGCATGTTAATCCACGTATTT CATCGCGAC	*Afl*II
alr-XbaI-Terma nitor-pR-FW	TGCTCTAGAGCAAAGCATAGGGTTTGCA GAATCCCTGCTTATATCTAACACCGTGC GTGT	*Xba*I

cells harbouring plasmid pET-28a(+) were treated in the same way. The crude extracts were used for activity determination.

The reaction mixture contained 0.5 mL 50 mM phenylpyruvate, 0.4 mL 50 mM D-Ala, 0.25 mL 200 μM pyridoxal phosphate and 3.85 mL crude extract (10–200 U/mL) in 100 mM Na-phosphate buffer (pH 7.5). The reaction was started by addition of crude extract, and terminated by addition of 400 μL phosphoric acid. The unit of Dat activity was defined as the amount of enzyme catalyzing phenylpyruvate to form 1 μmol D-Phe per min under the assay conditions. Products were analyzed by HPLC.

Enzyme purification. The purification process was carried out by AKTA purifier 100 and the HisTrap affinity columns (Ni-Sepharose High Performance columns designed for high-resolution purification of histidine-tagged proteins, 17-5247-01, GE Healthcare BioSciences, Sweden) in a linear gradient elution with 10–100% elute buffer (100 mM Na-phosphate buffer (pH 7.5) with 0.5 M NaCl and 0.2 M imidazole). The peaks containing the target protein were characterized by SDS-PAGE [16] and Dat activity analysis. Protein concentration was determined by Bradford method [17] with BSA as a standard.

Characterization of Dat. The temperature optimum and thermostability of Dat activity were performed using crude extract of E. coli BL21(DE3) with the target plasmid in 100 mM Na-phosphate buffer (pH 7.5). The remaining enzyme activities were measured at 37°C after being cooled down. The optimum pH and pH stability were also measured in 100 mM Na-phosphate buffer with different pH values (from 5.4 to 9.0). Relative Dat activity was calculated from the activity of enzyme in 100 mM Na-phosphate buffer (pH 7.5) at 37°C as a standard.

The reaction mixtures with different final concentrations of phenylpyruvate were prepared to determine the kinetic parameters of Dat. Michaelis-Menten equation was applied to calculate K_m, and deduce k_{cat}. All the experiments were performed with 3 replicates and error was within 5%.

Construction of artificial D-Phe synthetic operon. The *dat* genes were PCR-amplified from plasmids $pETdat_{BS}$, $pETdat_{BL}$, or $pETdat_{BA}$, and ligated with pSY130-14 under a temperature-induced promoter p_R to regulate the expression of *dat*. These 3 genes containing promoter p_R were ligated with a plasmid pR15ABK resulting in L-Phe producing [7] to construct the plasmids pR15ABKpRdat. The *alr* (alanine racemase, EC: 5.1.1.1) gene amplified from E. coli W3110 was ligated with p_R, and then integrated into pR15ABKpRdat to construct D-Phe synthetic operon pR15ABKApRdat.

Fermentation of glucose to D-Phe in flasks and 15-L jar fermenter. Seventy mL of fermentation medium [18] in 500-mL conical flasks were inoculated by 5% seed and cultured at 33°C for 4–5 h (early stage of log phase). Then the temperature was elevated to 38°C to induce the expression of target genes. Inactivation of a branched-chain aminotransferase gene, *ilvE*, resulted in partial inhibition of the catabolism of Leu, Ile, and Val [19], these amino acids were supplemented in the fermentation medium. In addition, in the fermentation medium for *aspC*$^-$ strain, Asp was also supplemented. Fed-batch fermentation was performed in a 15-L jar fermenter with an initial broth volume of 6 L supplied with L-Val, L-Leu and Ile (1 g/L of each). The fermentation conditions were described in previous manuscript [6].

Analysis of dry cell weight and glucose, acetic acid, phenylpyruvate, and D/L-Phe content in fermentation medium. The cell concentration was measured as OD_{600} after appropriate dilution, where the dry cell weight (DCW) was calculated as follows: DCW (g/L) = $OD_{600} \times 0.3809 - 0.0048$ [6]. Glucose and acetic acid concentrations were assayed as previously described [20]. Phenylpyruvate was determinated by HPLC using a reverse C18 column (ODS; ID 5 μm; 250 mm × 4.6 mm, Sandyet Co., Ltd, China) and detected at 210 nm. The mobile phase was 20 mM Na-phosphate buffer (pH 7.5) : methanol (90 : 10, vol/vol). D/L-Phe were separated on Astec CLC-L Copper Ligand Exchange Column (150 × 4 mm, Sigma-Aldrich Co., Ltd.) with 5 mM copper sulfate : methanol (90 : 10, vol/vol), and detected at 254 nm. All the experiments were performed at least 3 replicates and error was within 5%.

RESULTS

Structure-based analysis of Dat. The 3D models of Dat from *B. subtilis* WB600, *B. licheniformis* ATCC 14580, and *B. amyloliquefaciens* were quite similar to the known Dat from *Bacillus* sp. The typical monomer of Dat was composed of 2 domains with different secondary and tertiary structure [21]. The small domain (N-terminal) consisted of amino acid residues 1–120, the remaining amino acid residues formed the large domain (C-terminal). The N-terminal domain consisted of 4 stranded antiparallel β-sheets. The strands 1 and 2 together with helix 1 formed a typical "Greek Key" structural motif. The long helices 3 and 4 inserted between strands 2 and 3 to cover one side of the N-terminal β-sheets to prevent the core of β-sheets from solvent molecules. The C-terminal domain had 2 mixed β-sheets (β-α-β motifs) which formed the pyridoxal phosphate(PLP)-binding sites. The N-terminal domain on one side of the C-terminal domain had a hydrophobic domain interface at which the cofactor PLP was bound [21]. On the other side of the C-terminal domain, 3 α-helices (helices 6–8) shielded this hydrophobic region. The residues Thr204, Thr240, Ile203 and Arg 50 formed hydrogen bonds with the three phosphate oxygen atoms of PLP.

Table 3. Dat activities from *B. subtilis* WB600, *B. licheniformis* ATCC 14580 and *B. amyloliquefaciens* expressed in *E. coli* BL21(DE3)

Enzyme	Total activity, U/mL	Specific activity, U/mg of protein, 10^{-3}	K_m, μM	k_{cat}, min^{-1}	K_{cat}/K_m, min^{-1} μM^{-1}
Dat_{BS}	142	245	2473	139	0.056
Dat_{BL}	18	35	3089	218	0.071
Dat_{BA}	103	467	2051	134	0.065

Computer-aided screening of Dat. Based on the 3D models of Dat and D-Phe, molecular docking was carried out to simulate the suitable configuration of Dat to formate D-Phe. For each Dat, 100 hits were examined to find out whether the appropriate active configuration was possible or not. In a suitable configuration, the D-Phe bound at a cavity above the PLP and the α-amino group of the D-Phe located closely enough to the C4' of PLP to form a covalent bond. The Lys-144 was supposed to be the key amino acid residue which facilitated the transamination reaction. The side chains of amino acid residues Ser239, Thr241, Ser242 and Ser179 formed a "hole" that might be the entrance of a pocket in which the side chain of the substrate could be caught and provided a surface where either nonpolar or polar side chains could interact. The side chain of D-Phe was automatically located near this hole. All the Dat showed this suitable configuration to formate D-Phe, thus we predicted they had the activity of producing D-Phe. Then these *dat* genes were amplified and heterologously expressed to characterize their capability on D-Phe formation.

Expression and purification of Dat. The functional expressions of *dat* from *B. subtilis, B. licheniformis*, and *B. amyloliquefaciens* were certified by detecting the Dat activity in crude extracts of IPTG-induced E. coli BL21 (DE3) cells. SDS-PAGE analysis of the soluble and insoluble fractions of crude extracts indicated that 20~28% of the total soluble protein was expressed as the appropriate protein. In the crude extract, the Dat_{BS} showed the highest activity (Table 3). As Dat would be the limiting step of D-Phe biosynthesis in an L-Phe producing chassis, the DatBS with highest activity was particularly promising for the production of this amino acid.

The temperature profile, thermostability and pH stability of recombinant enzymes. The optimum temperature for the recombinant enzymes was around 45°C (Fig. 1), which was much higher than the fermentation temperature of E. coli (33–38°C). Enzyme activity was measured after incubating for a certain time (10–120 min) to confirm the thermostability of Dat. All the enzymes were stable below 50°C (Fig. 2) which was much higher than the fermentation temperature. Dat_{BS} and Dat_{BA} were not stable after incubating at 55°C for 120 min, while more than 60% of Dat_{BL} activity was retained under these conditions.

All the Dat in 100 mM Na-phosphate buffer with different pH were placed at 4°C for a certain time (0–150 h) before activity determination. The enzymes were stable at pH 7.5–8.0 for 150 h (Fig. 3) indicating that all the Dat were stable at alkalescent environment. The relative enzyme activity decreased lower than 50% under pH 6.6 showing that Dat was unstable under acidic conditions.

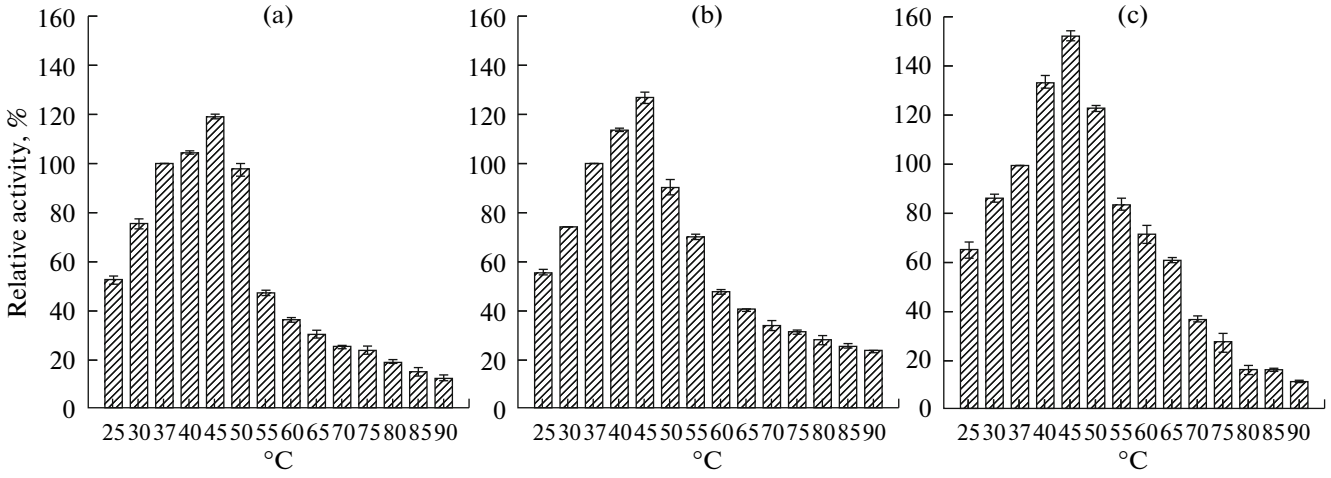

Fig. 1. The effect of temperature on the acitivity of Dat_{BS} (a), Dat_{BL} (b), Dat_{BA} (c).

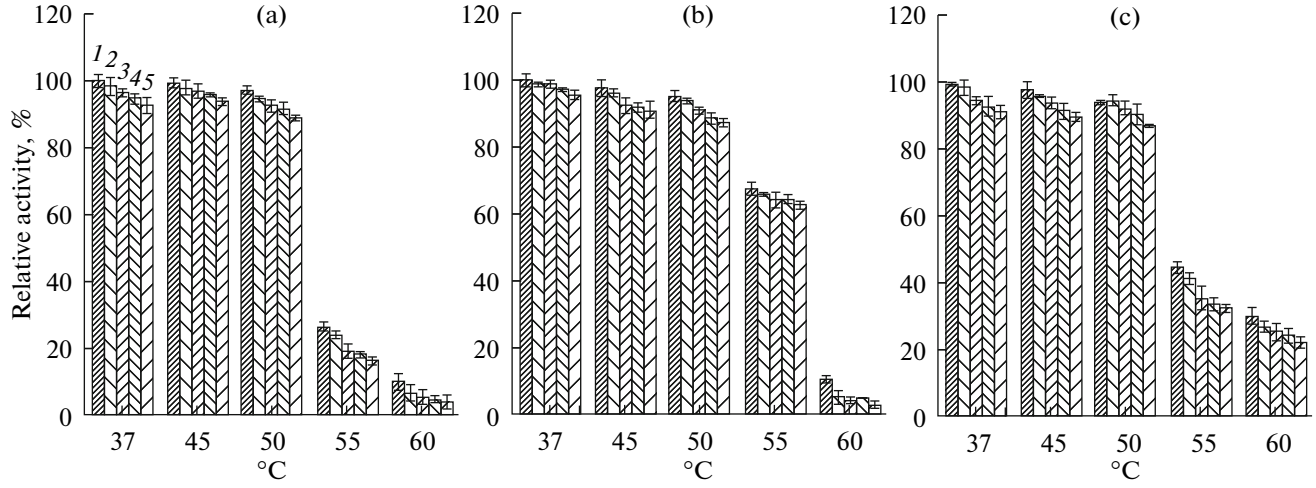

Fig. 2. The thermalstability of Dat_{BS} (a), Dat_{BL} (b), Dat_{BA} (c) after incubation for 10 (*1*), 30 (*2*), 60 (*3*), 90 (*4*) and 120 min (*5*).

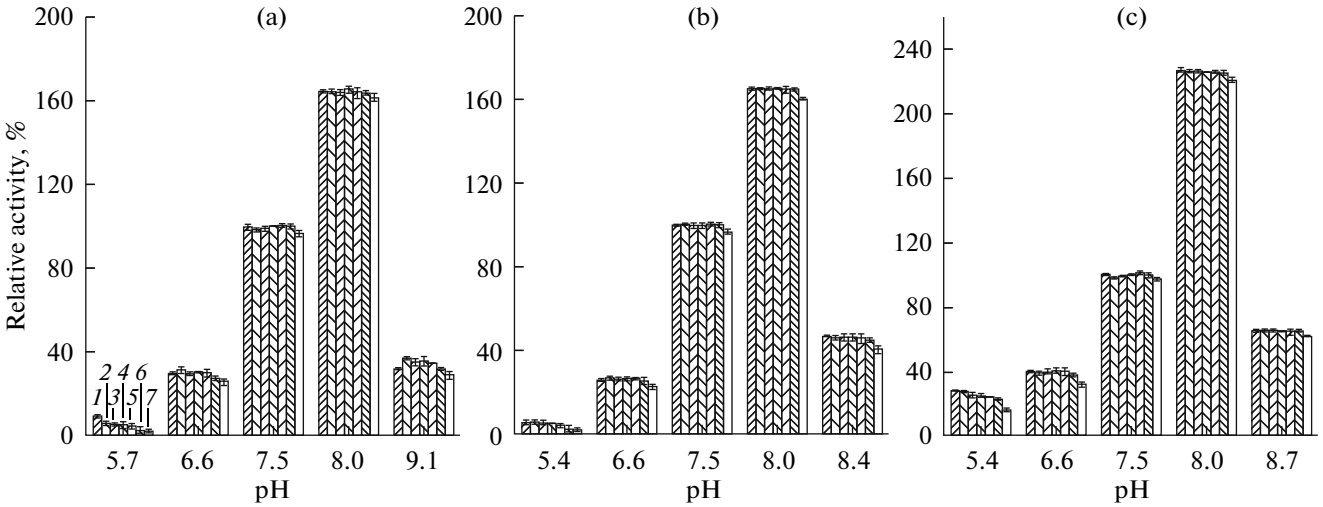

Fig. 3. The pH stability of Dat_{BS} (a), Dat_{BL} (b), Dat_{BA} (c) after 0 (*1*), 18 (*2*), 30 (*3*), 60 (*4*), 90 (*5*), 120 (*6*) and 150 h (*7*).

Characterization of the kinetic parameters of Dat. The kinetic parameters of Dat are shown in Table 3. The Dat_{BA} had the highest specific activity, whereas the lowest k_{cat}. The expression efficiency of Dat_{BA} was much lower than that of Dat_{BS} because Dat_{BS} (with lower specific activity) showed a higher total activity than Dat_{BA}.

Overexpression of *dat* in the L-Phe producing chassis. To produce D-Phe from glucose, a system level engineered strain E. coli W14 (pR15ABK) to increase L-Phe metabolic flux was used. The plasmid pR15ABK was used to overexpress *aroG15* (DAHP-synthase), $pheA^{fbr}$ (chorismate mutase and prephenate dehydratase), *ydiB* (shikimate dehydrogenase) and *aroK* (shikimate kinase) [7] under the control of temperature-induced promoter to increase the synthesis of phenylpyruvate. In addition, the expression of target genes was switched on or off efficiently and rapidly by a simple temperature change.

As the production of D-Phe in lag phase would block the cell growth, the expression of dat_{BS}, dat_{BL}, and dat_{BA} was also controlled by temperature-induced promoter p_R and the three *dat* genes were ligated with pR15ABK. The functional expression of different *dat* was confirmed by detecting the corresponding enzyme activities in the cell-free extract (Fig. 4). The extract of *E. coli* W14 (pR15ABKpRdat_{BS}) accumulated most D-Phe in the reaction mixture, while there was no D-Phe in the fermentation broth. It was assumed that the amino transfer process should be a limited step as the *E. coli* W14 (pR15ABKpRdat_{BS}) has an efficient phenylpyruvate synthetic pathway. Thus, the effects of the aminotransferase activities of TyrB, AspC, IlvE

and the supplement of amino donor on D-Phe synthesis were further analyzed.

Increasing the supply of amino donor D-Ala. The effect of supplement of amino donor on D-Phe synthesis was studied. After supplementing 1 g/L D-Ala in the fermentation medium, *E. coli* BCEA (pR15ABKpRdat$_{BS}$) accumulated 229 mg/L D-Phe in 48 h, *E. coli* BCEA (pR15ABKpRdat$_{BL}$) and *E. coli* BCEA (pR15ABKpRdat$_{BA}$) produced 179 mg/L and 57 mg/L D-Phe, respectively. This result demonstrated that D-Ala supply was a limiting node in D-Phe biosynthesis, thus gene *alr* encoding an alanine racemase was also expressed by pR15ABKpRdat to supply D-Ala by the strain itself.

After the overexpression of *dat* and *alr* genes, significant amount of D-Phe (231 mg/L) was detected in the fermentation broth of *E. coli* BCEA (pR15ABKApRdat$_{BS}$) without addition of D-Ala (Table 4). BCEA (pR15ABKApRdat$_{BL}$) and BCEA (pR15ABKApRdat$_{BA}$) had a lower yield of D-Phe than BCEA (pR15ABKApRdat$_{BS}$) (Table 4).

The effect of TyrB, AspC, IlvE and DadA on D-Phe formation. Three aminotransferases, TyrB, AspC, and IlvE, are involved in the last step of L-Phe synthesis [22], the disruption of TyrB, AspC, and IlvE might promote phenylpyruvate supply for D-Phe. After the disruption of L-tyrosine aminotransferase, the accumulation of D-Phe gradually increased (Table 5), and the *E. coli* BCE (pR15ABKApRdat$_{BS}$), deficient in *ilvE*, *tyrB* and *aspC*, accumulated 219 mg/L D-Phe which was 842-fold of the parent strain *E. coli* W14 (pR15ABKApRdat$_{BS}$).

D-amino acid dehydrogenase (DadA) catalyzes the oxidative deamination of most D-amino acids [23]. Then the *dadA* gene was deleted to construct E. coli mutants BCA and BCEA. Although these mutants displayed lower growth ability in comparison to wild-type *E. coli* W14, the D-Phe yield of BCA (pR15ABKApRdat$_{BS}$) and BCEA (pR15ABKApRdat$_{BS}$) continuously increased compared to BC (pR15ABKApRdat$_{BS}$) and BCE (pR15ABKApRdat$_{BS}$). Lowest L-Phe concentration was achieved and phenylpyruvate was slightly lower than that of BCE (pR15ABKApRdat$_{BS}$) and BC (pR15ABKApRdat$_{BS}$) (Table 5).

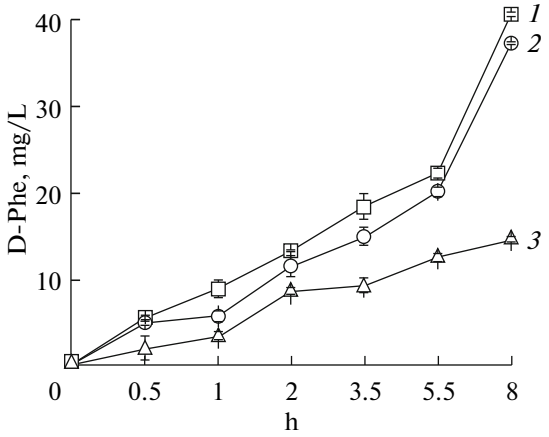

Fig. 4. Dat activity of E.coli W14 (pR15ABKpRdat$_{BS}$) (*1*), *E. coli* W14 (pR15ABKpRdat$_{BL}$) (*2*) and *E. coli* W14 (pR15ABKpRdat$_{BA}$) (*3*).

The production of D-Phe from glucose in a 15-L jar fermenter. Based on above study, the strain *E. coli* BCEA (pR15ABKApRdat$_{BS}$) was chosen to assess D-Phe production in a 15-L jar fermenter. As it was aspartate and branched-chain amino acids auxotroph, the growth was slower and stopped at 48 h of incubation at 33°C. D-Phe had not accumulated until the fermentation temperature was elevated to 38°C. After 60 h of fermentation time, 1.72 g/L D-Phe was synthesized, meanwhile 3.86 g/L of L-Phe and 137 mg/L of phenylpyruvate were detected (Fig. 5).

DISCUSSION

D-Phe is an important side-chain building block in the fine chemicals industry. It would be of environmental and commercial interest to develop a fermentative procedure producing D-Phe from renewable and sustainable resources. In this study, the fermentation of D-Phe was achieved by replacing L-aminotransferase with D-isomer in an L-Phe producing chassis.

A prerequisite for the artificial D-Phe biosynthetic pathway was the screening of enzyme capable of converting phenylpyruvate into D-Phe. The 3D models of Dat from *B. subtilis, B. licheniformis*, and *B. amyloliquefaciens* were constructed, and then their catalytic activities to formate D-Phe were predicted by vir-

Table 4. D-Phe, L-Phe and phenylpyruvate produced by *E. coli* BCEA with different plasmids after incubation at 38°C for 48 h in conical flasks

Strain	Concentration, mg/L		
	D-Phe	L-Phe	phenylpyruvate
E. coli BCEA (pRl 5ABKApRdat$_{BS}$)	231	125	206
E. coli BCEA (pR15ABKApRdat$_{BL}$)	182	161	274
E. coli BCEA (pR15ABKApRdat$_{BA}$)	60	179	359

Table 5. D-Phe, L-Phe and phenylpyruvate production by different *E. coli* strains after incubation at 38°C for 48 h in conical flasks

Strain	Concentration, mg/L		
	D-Phe	L-Phe	phenylpyruvate
E. coli W14 (pR15ABKApRdat$_{BS}$)	0.26	858	198
E. coli W14B (pR15ABKApRdat$_{BS}$)	2.00	877	159
E. coli BC (pR15ABKApRdat$_{BS}$)	93.00	203	466
E. coli BCE (pR15ABKApRdat$_{BS}$)	219.00	161	226
E. coli BCA (pR15ABKApRdat$_{BS}$)	141.00	208	320
E. coli BCEA (pR15ABKApRdat$_{BS}$)	233.00	123	205

tual screening. Then their functions were confirmed by in vitro enzyme activity determination using cell-free extracts from *E. coli* BL21 (DE3) expressing *dat* genes (Table 3). All the heterogenous Dat displayed high thermostability and pH stability, and the Dat$_{BS}$ showed higher activity than Dat$_{BL}$ and Dat$_{BA}$.

The *dat* genes were ligated with temperature-controlled promoter p_R, and the transamination of phenylpyruvate to D-Phe was confirmed by in vitro formation of D-Phe from phenylpyruvate. The utilization of temperature-controlled promoter could separate the cell growth phase and the product accumulation phase, thus, it decreased the negative effect of product synthesis on cell growth. Meanwhile, the D-Phe was not detectable in the fermentation broth, thus, the D-Phe synthesis was further increased by increasing the amino donor supply and deleting the L-tyrosine aminotransferase gene.

Although wild-type *E. coli* could produce D-Ala via host-synthetised L-Ala using the catabolic alanine racemase, it was proved that the D-Ala was not enough for D-Phe synthesis, and the supplement of D-Ala was necessary for D-Phe production. Then an alanine racemase encoded by the *alr* gene was co-expressed with *dat* gene to increase the supply of D-Ala. Integrating the expression of *dat* and *alr* into an *E. coli* strain optimized for the production of phenylpyruvate resulted in fermentative production of D-Phe, demonstrating the biosynthetic pathway of D-Phe was constructed successfully. The extracellular accumulation of D-Phe indicated that D-Phe could be secreted by endogenous exporters or diffusion without further strain improvement.

The inactivation of L-tyrosine aminotransferase was firstly proved to be an effective strategy for D-Phe accumulation. As the deletion of L-tyrosine aminotransferase gene *tyrB*, the D-Phe concentration gradually increased. After the deletion of *ilvE*, *tyrB* and *aspC* genes, the *E. coli* BCE (pR15ABKApRdat$_{BS}$) accumulated 219 mg/L D-Phe which increased the yield of D-Phe by 842-fold compared to the parent strain E. coli W14 (pR15ABKApRdat$_{BS}$). By the deletion of *dadA* gene, the accumulation of D-Phe was further increased to 233 mg/L. The D-Phe fermentation was carried out in a 15-L jar fermenter with a final production of 1.72 g/L, which was much higher than the previous concentration (1.12 g/L) [8]. In this paper, we used 3 different strategies to obtain a higher D-Phe production: the screening of high efficiency Dat, the high

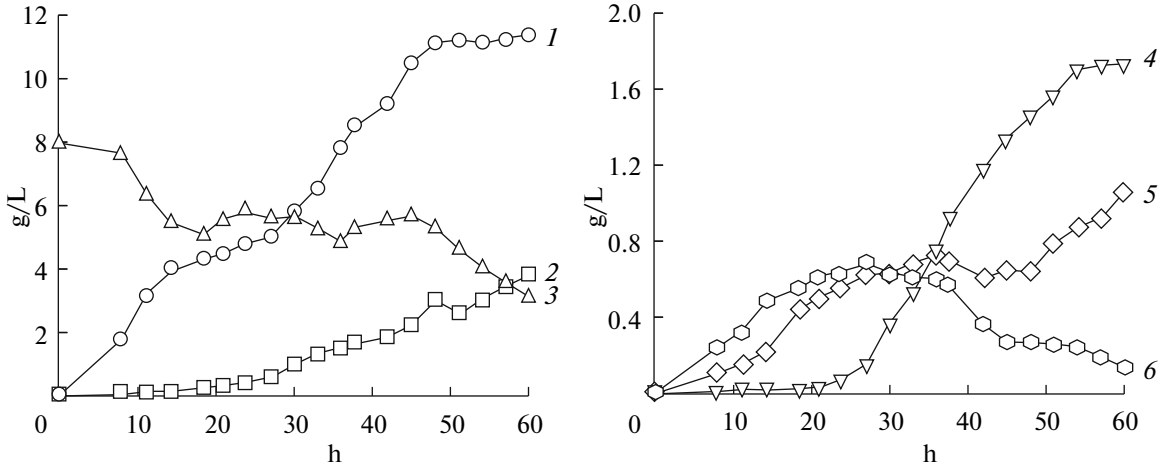

Fig. 5. Profiles of DCW (*1*) and L-Phe (*2*), glucose (*3*), D-Phe (*4*), acetic acid (*5*) and phenylpyruvate (*6*) concentration during *E. coli* BCEA (pR15ABKApRdat$_{BS}$) fermentation in a 15-L jar fermenter.

influx of the phenylpyruvate pathway, and the rigorous controlling of gene expression. We firstly compared the characterization of *dat* from different strains to screen an efficient Dat. Then the high L-Phe producing chassis was engineered to be a phenylpyruvate supplier to provide sufficient precursor for D-Phe biosynthesis. At last, the expression of target genes was segregated with cell growth to decrease the negative effect on cell metabolism. The engineered pR15ABKApRdat$_{BS}$ using the bacteriophage λ promoter p_R (for *aroG15*, *dat*, *alr*) and p_L (for *pheA*^{fbr}, *ydiB*, *aroK*) enables a simple temperature change to efficient and rapid switch on or off the expression of target genes. Switching off D-Phe generation during lag phase could improve biomass accumulation and intensifying target genes expression after the genetic switch on could promote D-Phe accumulation during the production phase.

The L-Phe accumulation was rather unpredictable, as in the extract of an *ilvE tyrB* and *aspC* triple mutant, no aminotransferase activity for the α-keto acids of L-Phe could be detected [24]. To eliminate the accumulation of L-Phe, the characterization and utilization of L-amino acid deaminase should be taken into consideration. L-amino acid deaminase can deaminate a wide range of L-amino acids to generate corresponding keto acids which is active on L-Phe but not on L-Ala [25]. Once L-Phe was deaminized, the product can serve as amino acceptor for D-Phe biosynthesis.

In this study, *dat* genes from different *Bacillus* strains were heterologously expressed and characterized. An effective Dat with high efficiency, thermostability, and pH stability was screened and used in the fermentative production of D-Phe. Then the supply of amino donor and the inactivation of L-tyrosine aminotransferase was studied to further increase the product yield. To our best knowledge, we have reported the highest D-Phe fermentation yield by building a functional multi-step pathway in *E. coli*.

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REFERENCES

1. Vedha-Peters, K., Gunawardana, M., Rozzell, J.D., and Novick, S.J., *J. Am. Chem. Soc.*, 2006, vol. 128, no. 33, pp. 10923–10929.
2. Tripathi, C.K.M., Bihari, V., and Tyagi, R.D., *Process Biochem.*, 2000, vol. 35, no. 10, pp. 1247–1251.
3. Wakayama, M., Yoshimune, K., Hirose, Y., and Moriguchi, M., *J. Mol. Catal. B: Enzym.*, 2003, vol. 23, no. 2, pp. 71–85.
4. Bae, H.S., Hong, S.P., Lee, S.G., Kwak, M.S., Esaki, N., and Sung, M.H., *J. Mol. Catal. B: Enzym.*, 2002, vol. 17, no. 6, pp. 223–233.
5. Bongaerts, J., Kramer, M., Muller, U., Raeven, L., and Wubbolts, M., *Metab. Eng.*, 2001, vol. 3, no. 4, pp. 289–300.
6. Liu, S.P., Xiao, M.R., Zhang, L., Xu, J., Ding, Z.Y., Gu, Z.H., and Shi, G.Y., *Process Biochem.*, 2013, vol. 48, no. 3, pp. 413–419.
7. Liu, S.P., Xiao, M.R., Liu, R.X., Zhang, L., Ding, Z.Y., Gu, Z.H., and Shi, G.Y., *Process Biochem.*, 2014, vol. 49, no. 5, pp. 751–757.
8. Taylor, P.P., Grinter, N.J., McCarthy, S.L., Pantaleone, D.P., Ton, J.L., Yoshida, R.K., and Fotheringhan, I.G., *ACS Symp. Series*, 2001, pp. 65–75.
9. Tanizawa, K., Masu, Y., Asano, S., Tanaka, H., and Soda, K., *J. Biol. Chem.*, 1989, vol. 264, no. 5, pp. 2445–2449.
10. Taylor, P.P. and Fotheringham, I.G., *Biochim. Biophys. Acta*, 1997, vol. 1350, no. 1, pp. 38–40.
11. Datsenko, K.A. and Wanner, B.L., *Proc. Natl. Acad. Sci. USA*, 2000, vol. 97, no. 12, pp. 6640–6645.
12. Benkert, P., Biasini, M., and Schwede, T., *Bioinformatics*, 2011, vol. 27, no. 3, pp. 343–350.
13. Guex, N. and Peitsch, M.C., *Electrophoresis*, 1997, vol. 18, no. 15, pp. 2714–2723.
14. Kobayashi, J., Shimizu, Y., Mutaguchi, Y., Doi, K., and Ohshima, K., *J. Mol. Catalysis B*, 2013, vol. 94, pp. 15–22.
15. Green, M.R. and Sambrook, J., *Molecular Cloning: A Laboratory Manual*, New York: Cold Spring Harbor, 2012.
16. Laemmli, U.K., *Nature*, 1970, vol. 227, no. 5259, pp. 680–685.
17. Bradford, M.M., *Anal Biochem.*, 1976, vol. 72, no. 1, pp. 248–254.
18. Gerigk, M.R., Maass, D., Kreutzer, A., Sprenger, G., Bongaerts, J., Wubbolts, M., and Takor, R., *Bioprocess Biosyst. Eng.*, 2002, vol. 25, no. 1, pp. 43–52.
19. Madsen, S.M., Beck, H.C., Ravn, P., Vrang, A., Hansen, A.M., and Israelsen, H., *Appl. Environ. Microbiol.*, 2002, vol. 68, no. 8, pp. 4007–4014.
20. Liu, S.P., Ding, Z.Y., Zhang, L., Gu, Z.H., Wang, X.L., Sun, X.J., and Shi, G.Y., *Biomass Bioenerg.*, 2012, vol. 37, pp. 237–242.
21. Sugio, S., Petsko, G.A., Manning, J.M., Soda, K., and Ringe, D., *Biochemistry*, 1995, vol. 34, no. 30, pp. 9661–9669.
22. Müller, U., Van Assema, F., Gunsior, M., Orf, S., Kremer, S., Schipper, D., and Wubbolts, M., *Metab. Eng.*, 2006, vol. 8, no. 3, pp. 196–208.
23. Wild, J. and Obrepalska, B., *Mol. Gen. Genet.*, 1982, vol. 186, no. 3, pp. 405–410.
24. Gelfand, D.H. and Steinberge, R.A., *J. Bacteriol.*, 1977, vol. 130, no. 1, pp. 429–440.
25. Baek, J.O., Seo, J.W., Kwon, O., Seong, S.I., Kim, I.H., and Kim, C.H., *J. Biotechnol.*, 2008, vol. 24, no. 12, pp. 2129.