Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supplementary Information

Switching two Nitrilase Regioselectivity toward Succinonitrile by Mutating the Active Center Pocket Key Residues Through a Semi-Rational Engineering

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Supplementary information

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1. Materials

Two recombinat strains harboring the bll6402NIT and blr 3397NIT both from *Bradyrhizobium japonicum* USDA110 were constructed using pET-28a as vector and stocked in our lab. *Escherichia coli* Bl21 was purchased from TransGen Biotech Co., Beijing, China. Succinonitrile and Succinic acid were purchased from Aladdin Co., China. 3-Cyanopropanoic acid was synthesized by Shao Yuan Co,. Shanghai, China. All the other chemicals were commercially available.

2. Sequence analysis

Firstly, bll6402NIT amino acids and other 10 nitrilases that could also convert succinonitrile to 3-cyanopropanoic acid were couducted multiple alignment using MEGA6 and GENEDOC. Secondly, bll6402NIT and blr3397NIT from same strain *Bradyrhizobium japonicum* USDA110 were conducted multiple aligment again by the same software. Modellering templete selection of bll6402NIT and blr397NIT was performed in the NCBI blastp using the Protein Data Bank database.

3. Homology modeling and Molecular docking

The three-dimensional structure model of wide type bll6402NIT and blr3397NIT and their mutants were generated by Modeller 9.19 using *Syechocystis sp*. PCC6803 nitrilase as the templete. Structures with the lowest DOPE scores were selected.[1] Model evaluation was performed using Verify 3D and the Ramachandran Plot web server.[2] The resulting model was used for further docking study. 3-Cyanopropanoic acid was docked into the active site of bll6402NIT, blr3397NIT and their mutants using Autodock4.2 program suite.[3] The structure of 3-cyanopropanoic acid was prepared and

subsequently MM2 minimized by Chem3D sofeware in Chem office15 suite. The dock performance was conducted with default parameters except for the number of runs 100. The grid box was set as a cube with the number of points in x,y,z dimension 40 and utlizing the Cys x,y,z spatial coordinates as the center. The dock results were prepared by Open Babel and Pymol edu.

4. Site-directed saturated mutagenesis

Site-directed saturated mutagenesis was performed using KOD-Plus Mutagenesis Kit (TOYOBO, Japan) according to the instruction manual. The mutant recombinant plasmids were constructed as follows: Inverse PCR was firstly performed to amplified the linear mutant recombinant plasmids by the following primers (bll6402NIT forward primer: 5'-TGCNNKGAGCACATTCAGCCGTTG-3', reverse frimer: 5'-GCACAAGGCTCCGAGACGTCCG-3'; blr3397NIT forward primer: 5'-TGCNNKGAGAACTACATGCCGATGCTGC-3', reverse primer: 5'-GATCACGGCGCCGATATTGCCGAGC-3', the underlined letters indicated mutant degenerate bases) using pET-28a-bll6402NIT and pET-28a-blr3397NIT as templete; *Dpn*I enzyme was then utlized to digest the templete pET28a-bll6402NIT and pET28a-blr3397NIT; *Dpn*I-treated PCR products were finally self-ligated by T4 Polynucleotide Kinase. The resulting reaction mixtures were transformed into *Escherichia coli* Bl21 competent cell according to the Molecular Cloning: A Laboratory Manual and positive colones were subsequently verified by sequencing.

5. Expression and purification of enzymes

For the expression of wide type bll6402NIT, blr3397NIT and their mutants, the

resulting recombinant *E. coli* cells were cultivated in Luria–Bertani liquid medium containing 50 mg/ml kanamycin at 37°C on a rotary shaker at 220 ×g. A final concentration of 0.1mM isopropyl-β-D-thiogalactoside was added for the induction when the optical density at 600 nm of the culture broth reached between 0.6 and 0.8. The cells were then further incubated at 37°C and 220 ×g for another 4-6 h. After centrifugation at 8,000 ×g for 10 min, the cells were harvested and preserved at -20°C for further experiments.

For the purification of wide type bll6402NIT, blr3397NIT and their mutants, Nickle affinity chromatography (Histrap HP column, GE Healthcare UK Ltd) was applied to purify the recombinant nitrilase by exploiting the histidine tag. The obtained cells were suspended and washed twice with 10 ml of sodium phosphate buffer (20 mM, pH 7.4). Then the cells were resuspended in 20 ml of the same buffer and disrupted by sonication on ice at 200 W for 10 min. The soluble fractions of the sonicated solution were obtained by centrifugation at 8,000 ×g for 30 min to remove the cell debris. The resulting supernatant was passed through a 0.22 μm filter, and then loaded onto a Ni-NTA column previously equilibrated with a binding buffer (20 mM sodium phosphate buffer, 500 mM NaCl, 10 mM imidazole, pH 7.4). The column was subsequently washed with 10 ml of wash buffer (20 mM sodium phosphate buffer, 500 mM NaCl, 50, 100 and 250 mM imidazole, pH 7.4) sequentially to wipe out the non-target proteins and eluted with the elution buffer (20 mM sodium phosphate buffer, 500 mM NaCl, 500 mM imidazole, pH 7.4). The purified enzymes were further ultrafiltrated-concentrated and then analyzed by 12% sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE). Protein concentration was determined using the Bradford method with bovine serum albumin as the standard.[4] All purification steps were performed at 4 °C.

6. Enzymatic assay and Kinetic constants

The standard enzymatic reaction toward succinonitrile and 3-cyanopropanoic acid were assayed in a reaction mixture (1ml) consisting of 100 mM sodium phosphate buffer (pH 7.2), 10 mM substrate and purified enzyme with a final concentration of 0.1 mg/ml. The reaction was performed at 30 °C for 24h and aliquots (100 μL) of the reaction mixture were taken at different time intervals and quenched with 100 μL HCl (1 M). The production of succinic acid and 3-cyanopropanoic acid was determined by HPLC analysis using a Zorbax SBaq column (4.6 mm × 250 mm, 5 μm) (Agilent Technologies, Ltd., USA) at a flow rate of 0.5 ml min^{-1}, and the mobile phase was acetonitrile and 5mM K_2HPO_4/ H_3PO_4, pH 2.9 (7:12, v/v).[5] The decrease in substrates and formation of products were measured at the absorption wavelength of 205 nm.

The kinetic constants of wide type bll6402NIT, blr3397NIT and their mutants toward succinonitrile and 3-cyanopropanoic acid were measured under standard assay condition with different substrate concentrations (1, 2.5, 5, 7.5, 10 and 20 mM). The results were calculated by Michaelis-Menten equation model. One unit of the enzyme activity was defined as the enzyme amount that produced 1 μmol succinic acid or 3-cyanopropanoic acid per minute under standard assay conditions. All measurements were conducted in triplicate in the present study and data were means ± standard deviations from three replications.

7. Molecular dynamics simulation

Molecular dynamics simulation was performed by Gromacs 4.6.5 with the GROMOS96 43A1 force field.[6] An external classical web server PRODRG (http://davapc1.bioch.dundee.ac.uk/cgi-bin/prodrg) was utilized to generate ligand-related files since gromacs does not have a force field for small molecules.[7] The docked enzyme-ligand complexes were solvated in the system of a dodecahedron box with a size of 1.0 nm and spc216 water was added. Sodium ions were then added to neutralize the system charge. A steepest descents minimization algorithm was utilized to perform energy minimization. The system was set to the equilibration phases for 100 ps using the NVT ensemble with a continuous temperature of 300 K and subsequently 100 ps using the NPT ensemble with the same temperature. Molecular dynamics simulation was run for 10 ns to conduct RMSD and Dc-s analysis.

8. Supplementary Tables and Figures

Tables

Table S1 Regioselectivity[a] of wild type bll6402NIT and blr3397 and their mutants towards 3-cyanopropanoic acid and succinic acid[b]

Enzyme	3-cyanopropanoic acid (%)	succinic acid (%)
bll6402NIT WT	100	0
A163D	0	100
A163E	0	100
A163H	0	100
A163N	0	100
A163Q	0	100
blr3397NIT WT	0	100
W172C	100	0
W172D	100	0
W172E	100	0
W172H	100	0
W172N	100	0
W172S	100	0

[a]Regioselectivity is presented as the percentage of the target product.
[b]Determination of target product by HPLC analysis.

Table S2 Distance (D_{c-s}) of Cys thiol S to the substrate cyano C

Enzyme	D_{c-s} (Å)
bll6402NIT WT	6.6
A163D	3.8
A163E	3.3
A163H	3.0
A163N	2.9
A163Q	3.5
blr3397NIT WT	3.5
W172C	7.6
W172D	7.0
W172E	7.7
W172H	8.5
W172N	5.2
W172S	7.2

Figures

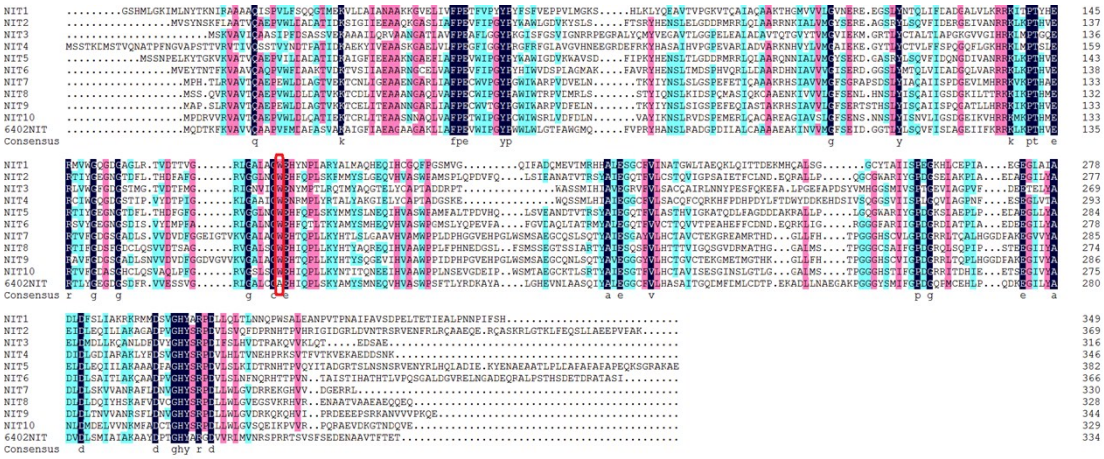


Figure S1 Multiple aligment between bll6402NIT amino acids and other 10 nitrilases that could also convert succinonitrile to 3-cyanopropanoic acid. The black portion represents the residues with 100% similarity; the pink portion represents the residues with 100%-75% similarity and the blue portion represents the residues with 75%-50% similarity. The red frame portion represented the amino acid distinction between bll6402NIT and other nitrilases.

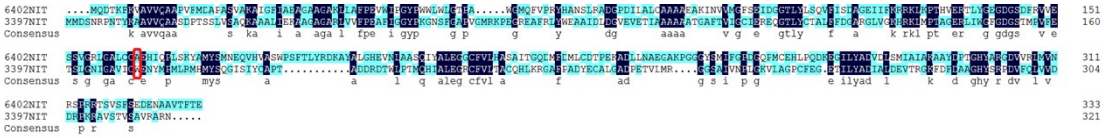


Figure S2 Sequence alignment of bll6402NIT and bll3397NIT amino acids. The black portion represents the residues with 100% similarity; and the blue portion represents the residues with 75%-50% similarity. The red frame portion represented the amino acid distinction between bll6402NIT and blr3397NIT.

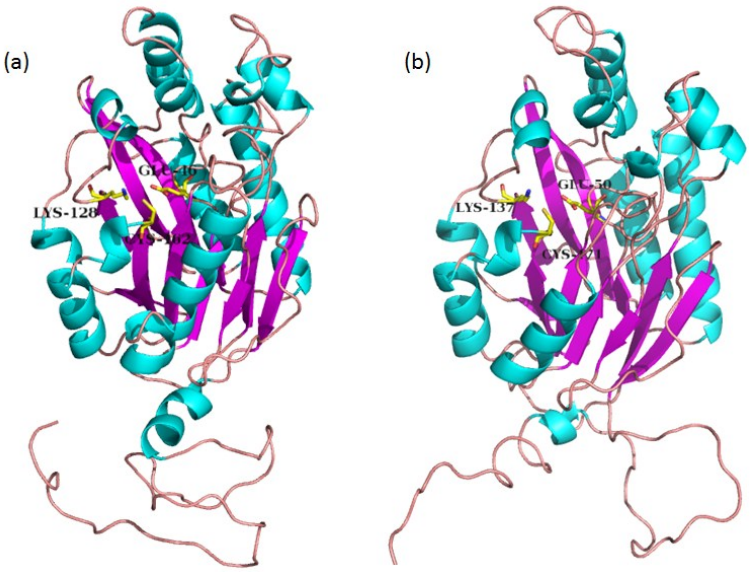
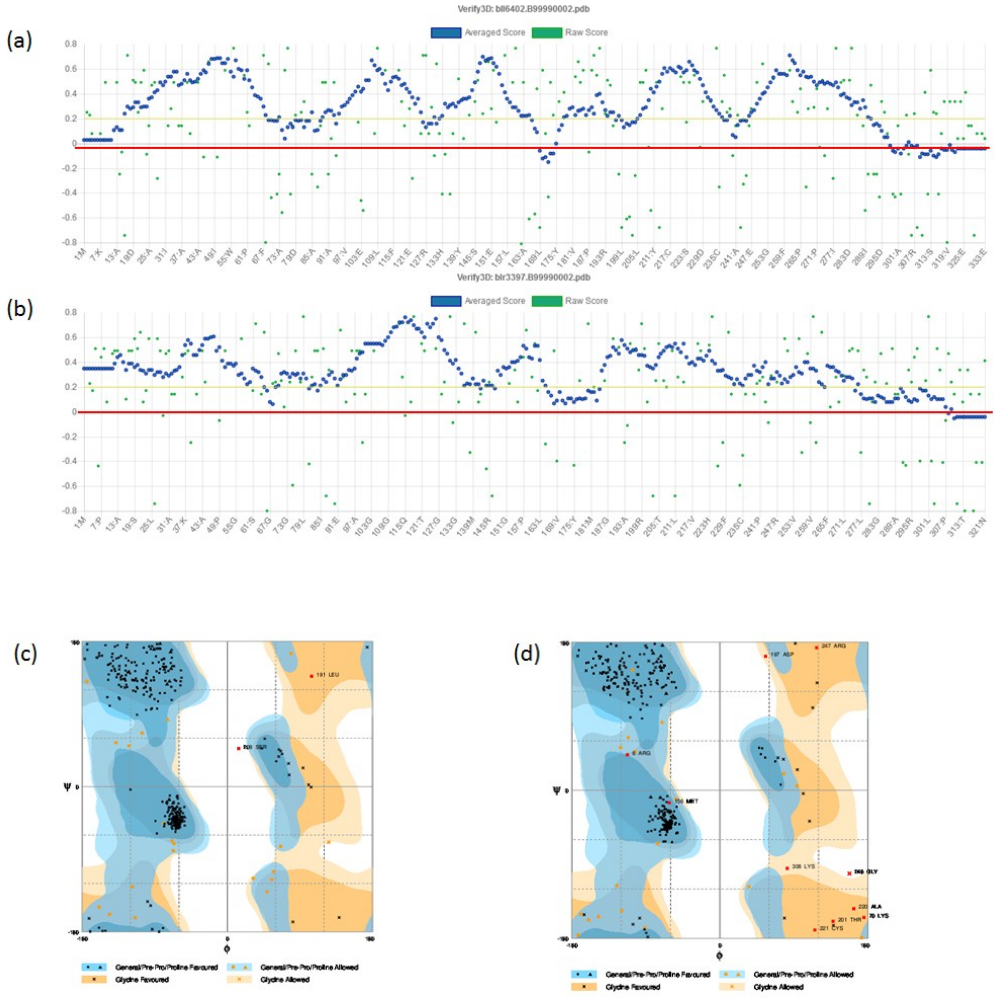


Figure S3 Constructed three-dementional structure of (a) bll6402NIT (b) and blr3397NIT. The catalytic triad (CEK) of bll6402NIT and blr3397NIT are shown in yellow stick.



Number of residues in favoured region: 309 (93.4%)
Number of residues in allowed region: 20 (6.0%)
Number of residues in outlier region: 2 (0.6%)

Number of residues in favoured region: 291 (91.2%)
Number of residues in allowed region: 18 (5.6%)
Number of residues in outlier region: 10 (3.1%)

Figure S4 Bll6402NIT (a,c) and blr3397NIT (b,d) model evaluations by Verify-3D and the Ramachandran Plot. The red line in (a) and (b) represents the percentage of amino acid with a 3D-1D above 0 for bll6402NIT and blr3397NIT.

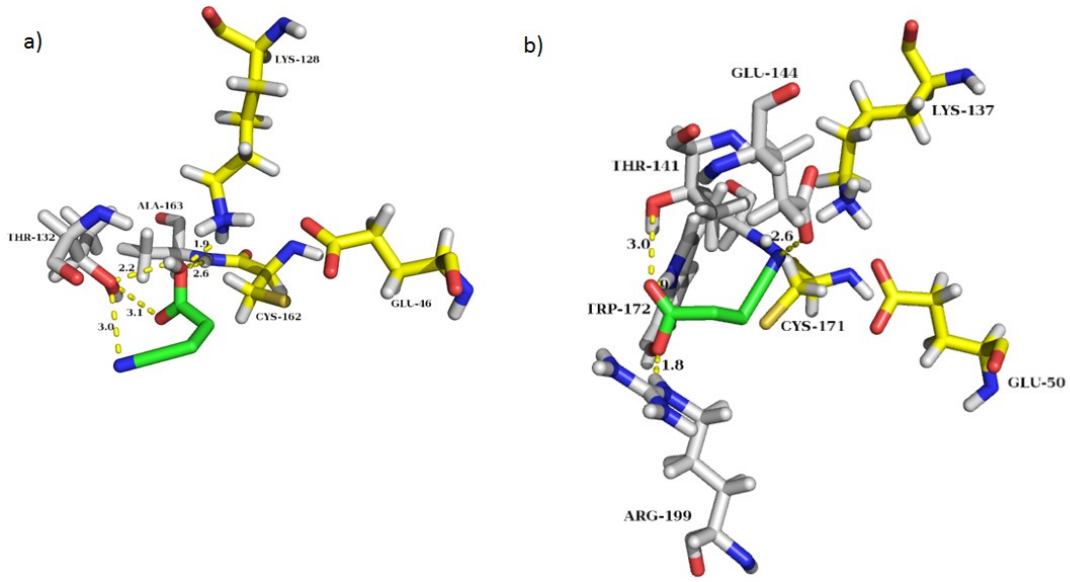


Figure S5 Autodock results between (a) bll6402NIT, (b) blr3397NIT and 3-cyanopropanoic acid. Amino acid around the ligand with 5 Å were picked up. For bll6402NIT-ligand complex, Thr132 and Ala163 were selected. For blr3397NIT-ligand complex, Thr141, Glu144, Trp172, Arg199 were selected. The yellow dash lines represents the hydrogen bond formed between ligand and selected amino acid.

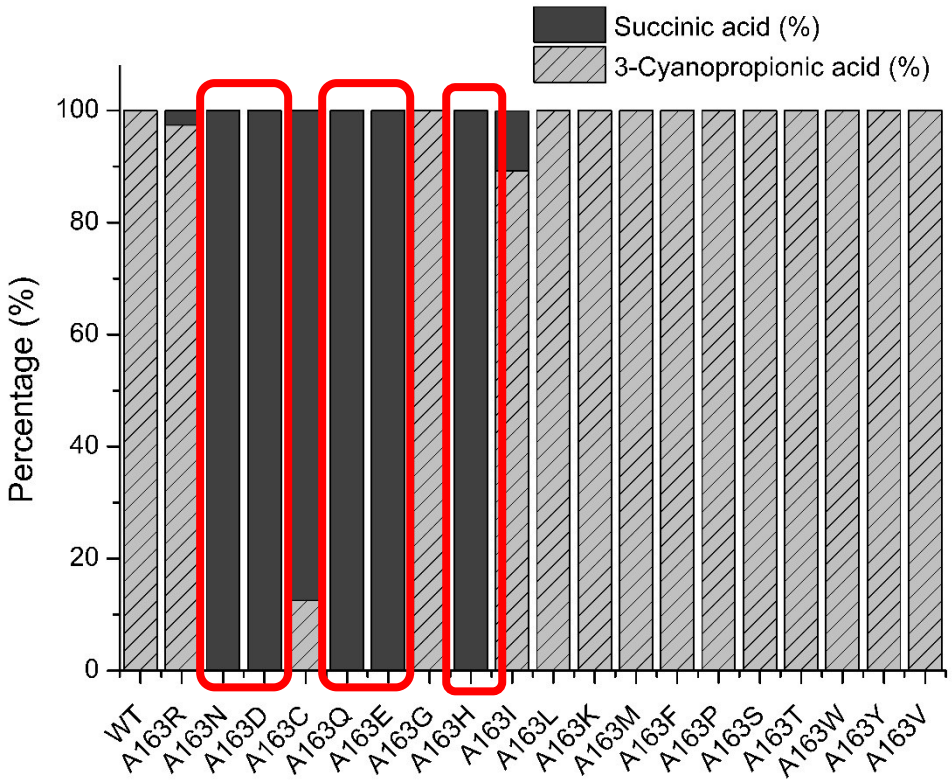


Figure S5 Conversion of succinonitrile by wild type Bll6402NIT and all saturated mutants. The red frame portion represents the mutants (A163N, A163D, A163Q, A163E, A163H) that regioselectivity switched.

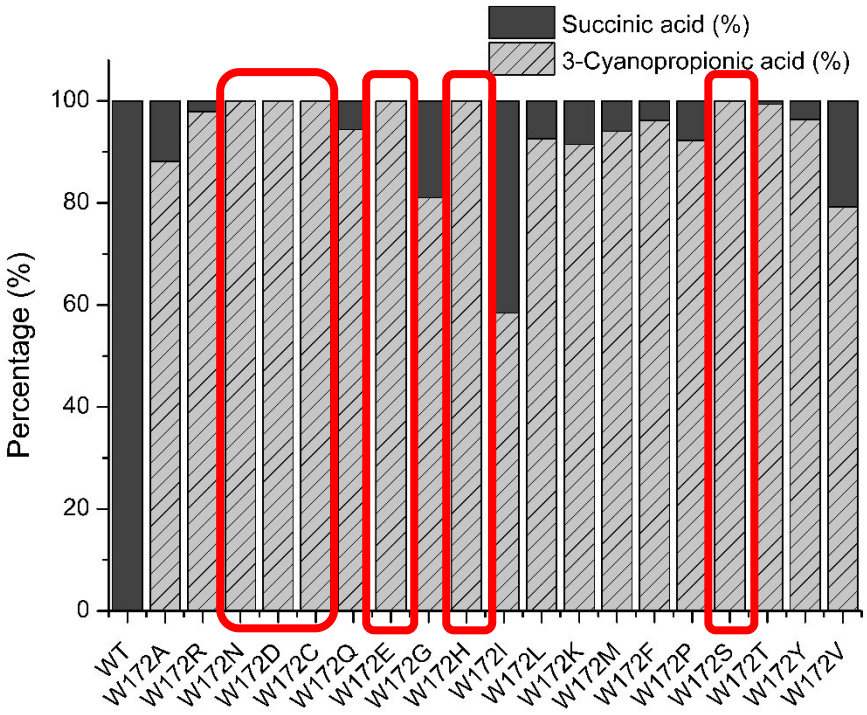


Figure S6 Conversion of succinonitrile by wild type blr3397NIT and all saturated mutants. The red frame portion represents the mutants (W172N, W172D, W172C, W172E, W172H, W172S) that regioselectivity switched.

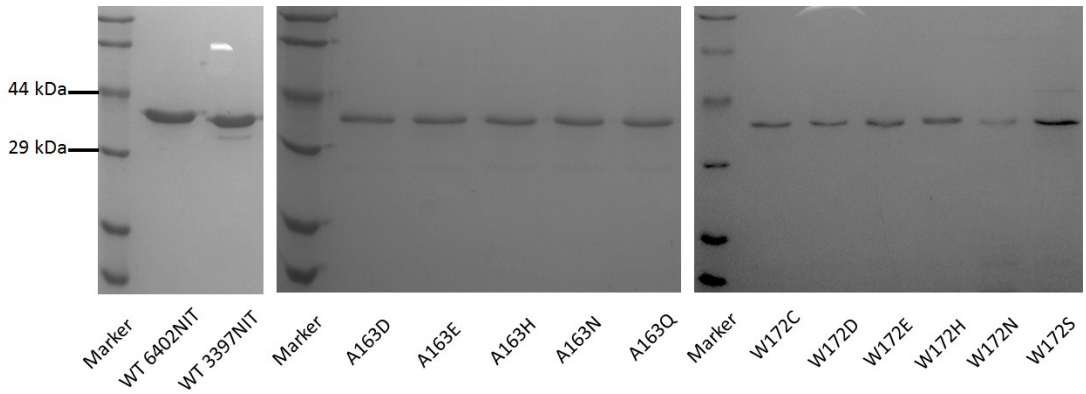


Figure S7 Pruification of wild type bll6402NIT and blr3397NIT and their mutants. Purification procedure was performed using nickel column according to the standard protein purification steps.

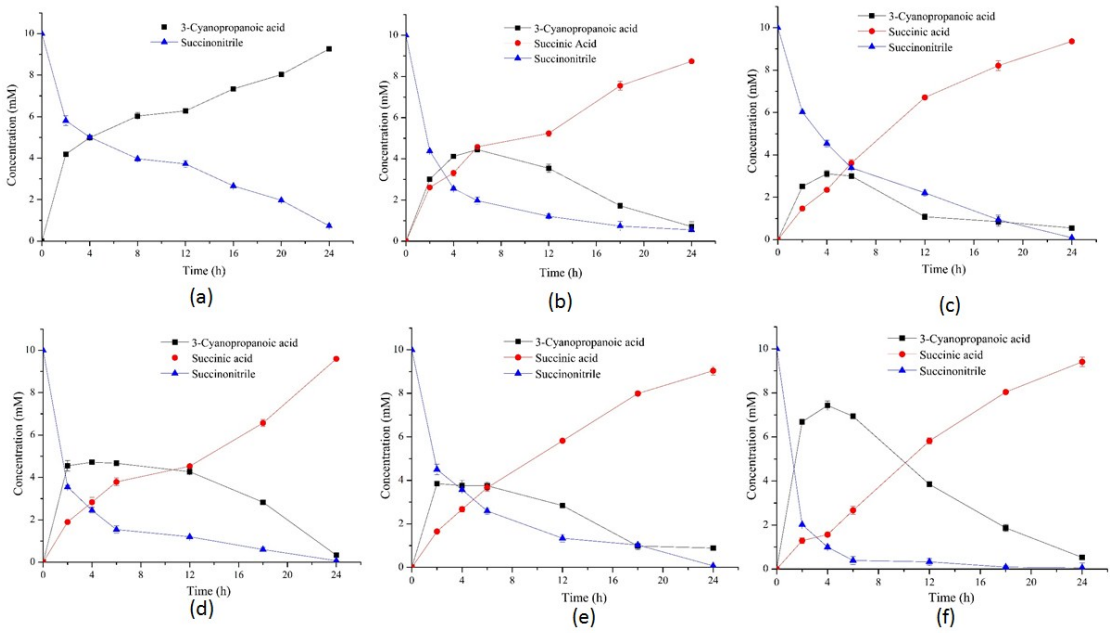


Figure S8 Time course conversion of succinonitrile by purified wild type bll6402NIT and its mutants. They were wild type bll6402 (a), mutants A163D (b), A163E (c), A163H (d), A163N (e), A163Q (f), respectively. Reaction mixture (1ml) consisting of 100 mM sodium phosphate buffer (pH 7.2), 10 mM substrate and purified enzyme with a final concentration of 0.1 mg/ml was performed to determine the time course conversion.

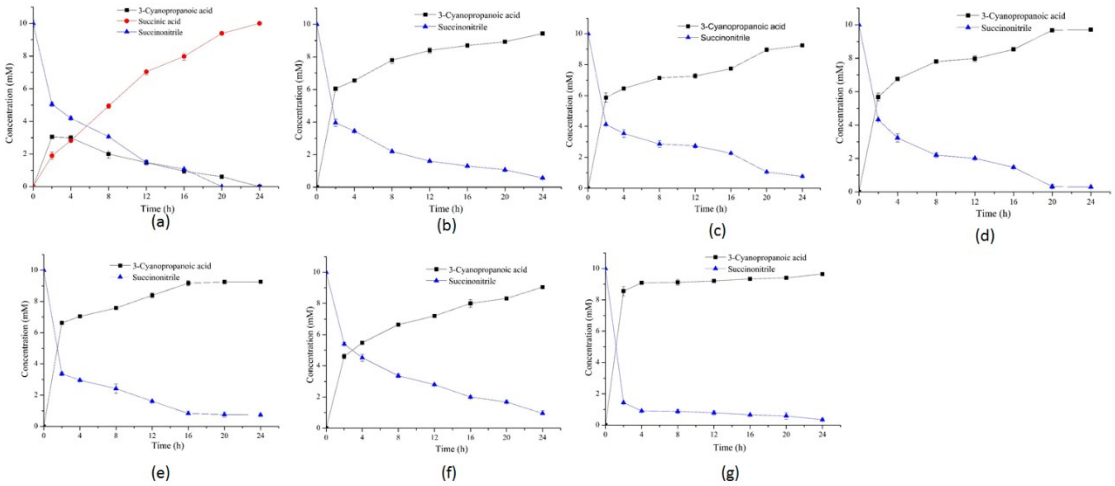


Figure S9 Time course conversion of succinonitrile by purified wild type blr3397NIT and its mutants. They were wild type blr3397NIT (a) and mutants W172C (b), W172D (c), W172E (d), W172H (e), W172N (f), W172S (g), respectively. Reaction mixture (1 ml) consisting of 100 mM sodium phosphate buffer (pH 7.2), 10 mM substrate and purified enzyme with a final concentration of 0.1 mg/ml was performed to determine the time course conversion.

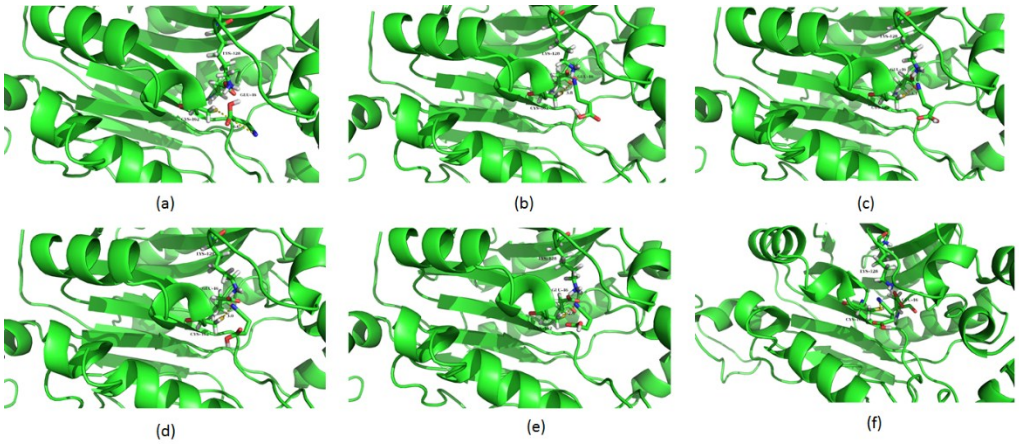


Figure S10 Dc-s measurement of wild type bll6402NIT and its mutants. They were wild type bll6402 (a), mutants A163D (b), A163E (c), A163H (d), A163N (e), A163Q (f), respectively. The measurement was peroformed using Pymol-edu.

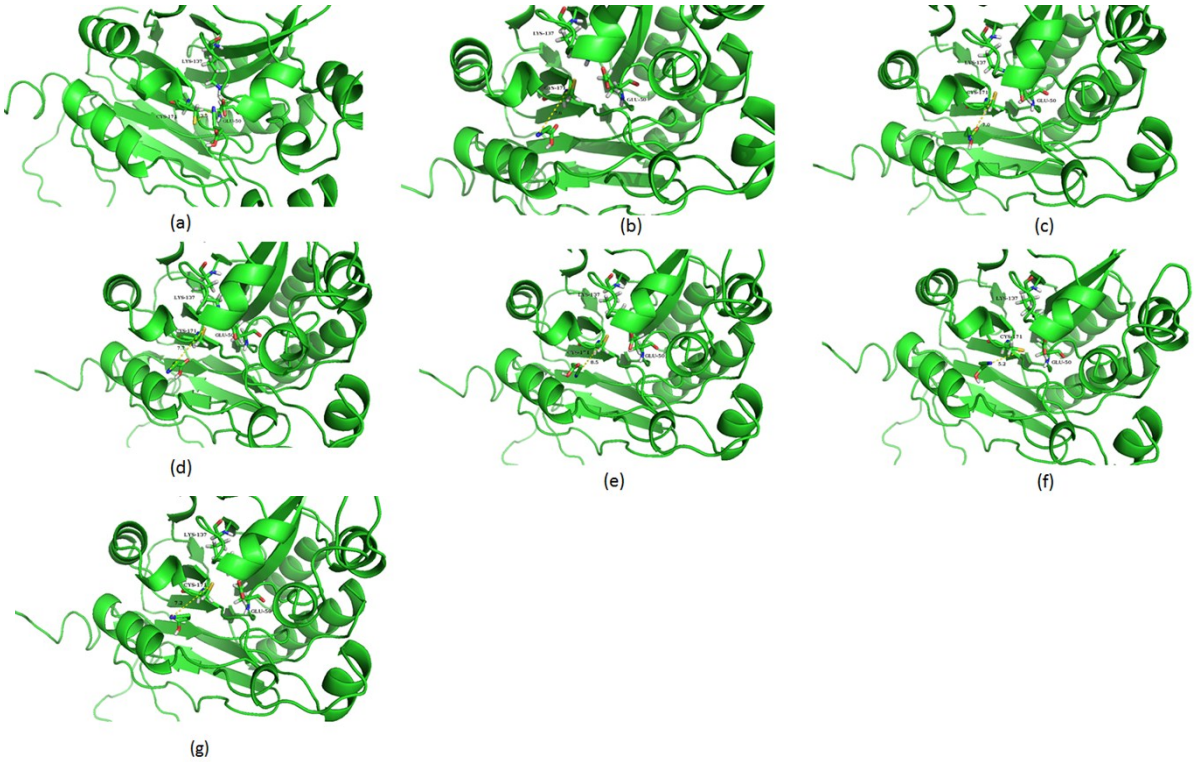


Figure S11 Dc-s measurement of wild type blr3397NIT and its mutants. They were wild type blr3397NIT (a) and mutants W172C (b), W172D (c), W172E (d), W172H (e), W172N (f), W172S (g), respectively. The measurement was peroformed using Pymol-edu.

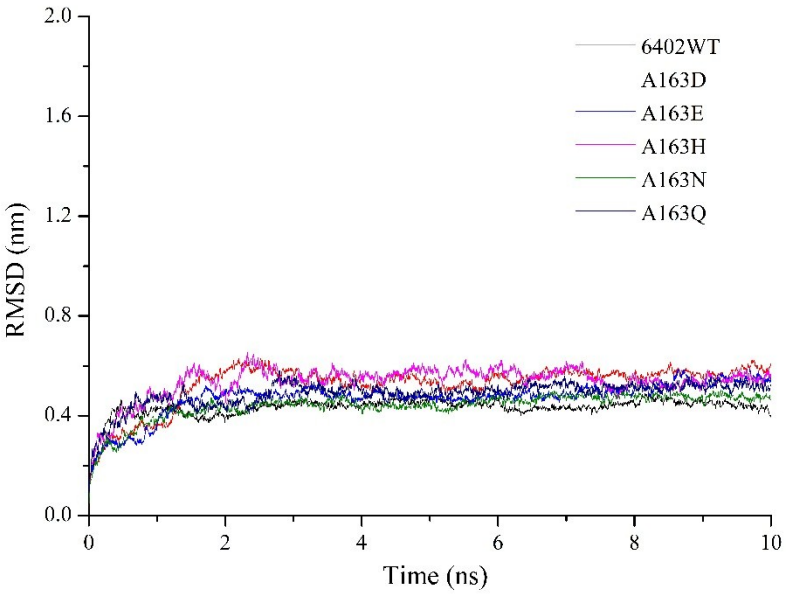


Figure S12 RMSD calculation of wild type bll6402NIT and its mutants during 10 ns molecular dynamic simulation.

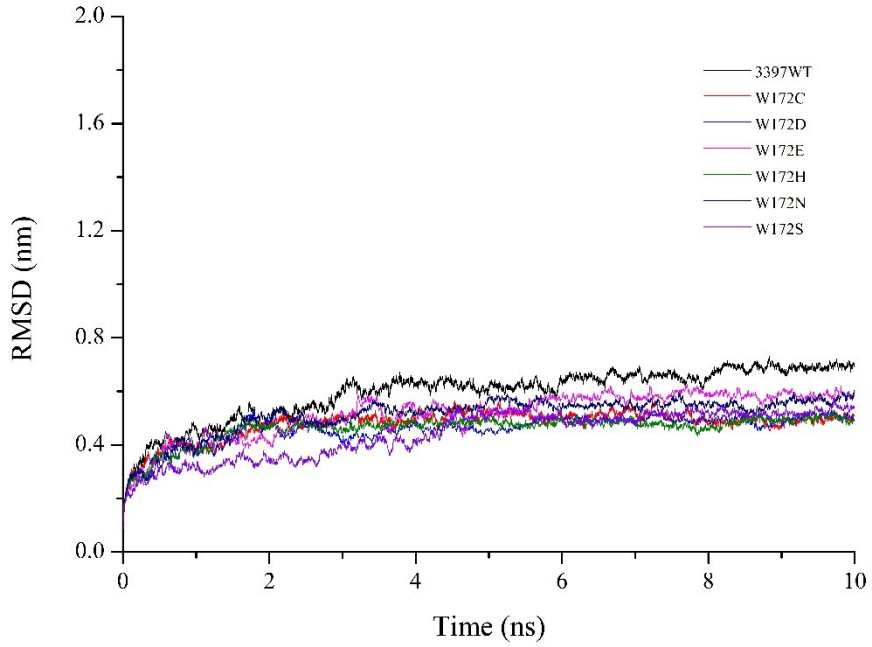


Figure S13 RMSD calculation of wild type blr3397NIT and its mutants uring 10 ns molecular dynamic simulation.

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