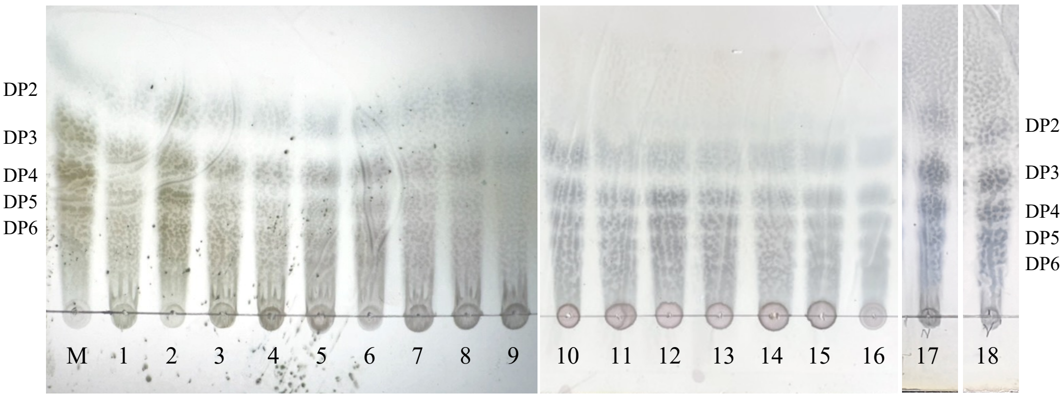
Supplementary Materials 1

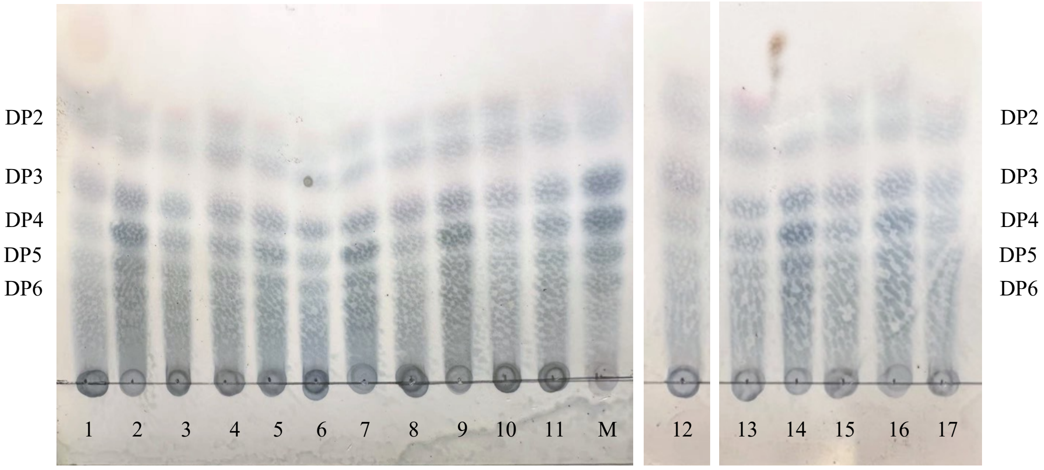
Thin-Layer Chromatography (TLC)

1 K172X-TLC



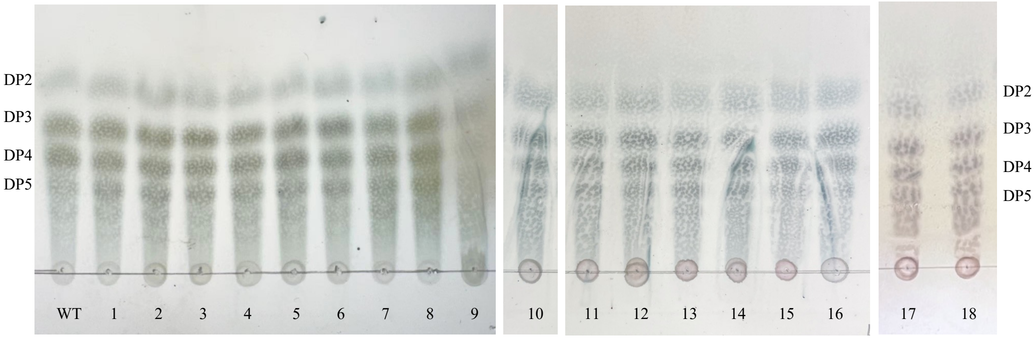
M、PyAly 1、K172D 2、K172Q 3、K172T 4、K172Y 5、K172A
6、K172S 7、 K172W 8、K172C 9、K172P 10、K172E 11、 K172F
12、 K172H 13、 K172I 14、K172L 15、 K172M 16、K172R 17、K172N
18、K172V

2 R159X-TLC



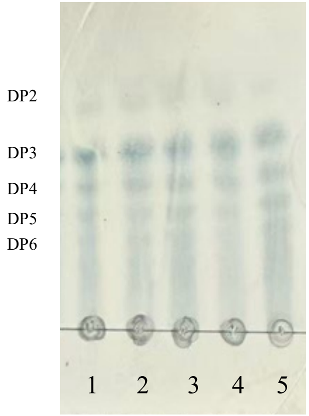
M. PyAly 1.R159I 2. R159S 3. R159L 4. R159H 5. R159M
6. R159A 7. R159N 8. R159P 9. R159T 10. R159Y 11. R159K
12. R159E 13.R159V 14. R159D 15. R159Q 16. R159C 17.R159F

3 R143X-TLC



WT. PyAly 1. R143M 2. R143W 3. R143Y 4. R143A 5. R143G 6. R143L 7. R143S
8. R143D 9. R143P 10. R143C 11. R143F 12. R143H 13. R143K 14. R143Q 15. R143T
16. R143V 17. R143I 18. R143N

4 R159-K172X -TLC



1. R159N-K172L
2.R159K-K172P
3. R159N-K172T
4. R159D-K172E
5. R159S-K172P

5 Reaction system

5.1 Table 1. Double -site mutant Reaction system

Mutant	Substrate (μL) / Enzyme (μL)
R159K-K172P	120 / 80
R159N-K172T	120 / 80
R159N-K172L	120 / 80
R159D-K172E	120 / 50
R159S-K172P	120 / 80

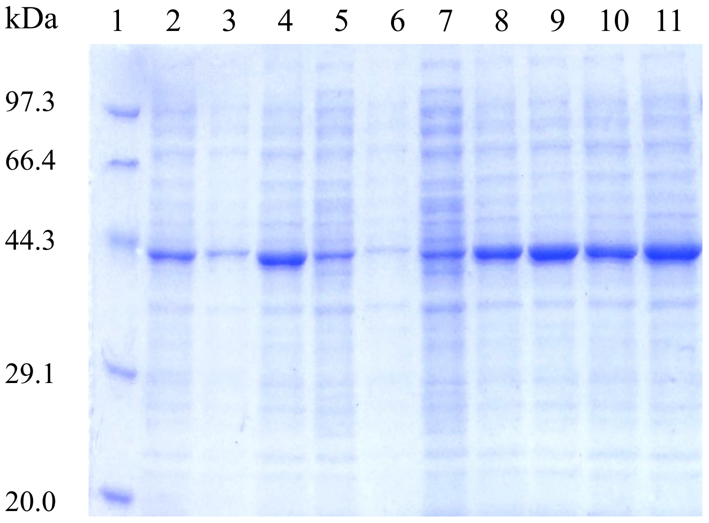
5.2 Table 2. Single-site mutant Reaction system

Mutant	Substrate (μL) / Enzyme (μL)	Mutant	Substrate (μL) / Enzyme (μL)	Mutant	Substrate (μL) / Enzyme (μL)
R159A	120 / 72	K172A	160 / 80	R143A	80 / 20
R159C	120 / 55	K172C	160 / 40	R143C	80 / 30
R159D	120 / 45	K172D	80 / 40	R143D	80 / 30
R159E	120 / 70	K172E	80 / 40	R143F	80 / 20
R159F	120 / 75	K172F	80 / 40	R143G	80 / 30
R159G	120 / 40	K172G	80 / 40	R143H	80 / 20
R159H	120 / 69	K172H	100 / 80	R143I	80 / 20
R159I	120 / 70	K172I	80 / 40	R143K	80 / 20
R159K	120 / 67	K172L	80 / 40	R143L	80 / 20
R159L	120 / 72	K172M	120 / 60	R143M	80 / 20
R159M	120 / 75	K172N	160 / 40	R143N	80 / 20
R159N	120 / 66	K172P	120 / 100	R143P	80 / 20
R159P	120 / 67	K172Q	100 / 100	R143Q	80 / 20
R159Q	120 / 60	K172R	80 / 40	R143S	80 / 20
R159S	120 / 50	K172S	80 / 40	R143T	80 / 20
R159T	120 / 65	K172T	80 / 40	R143V	80 / 20
R159V	120 / 72	K172V	80 / 40	R143W	80 / 120
R159Y	120 / 75	K172W	80 / 40	R143Y	80 / 120
		K172Y	80 / 40		

Supplementary Materials 2

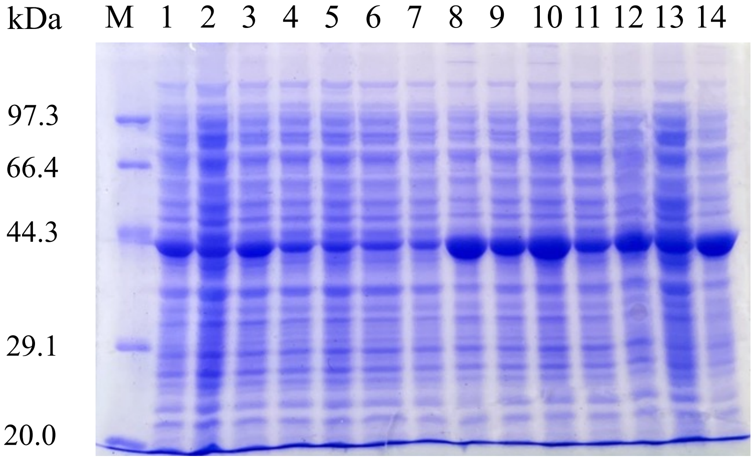
Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

1 6.5 SDS-PAGE



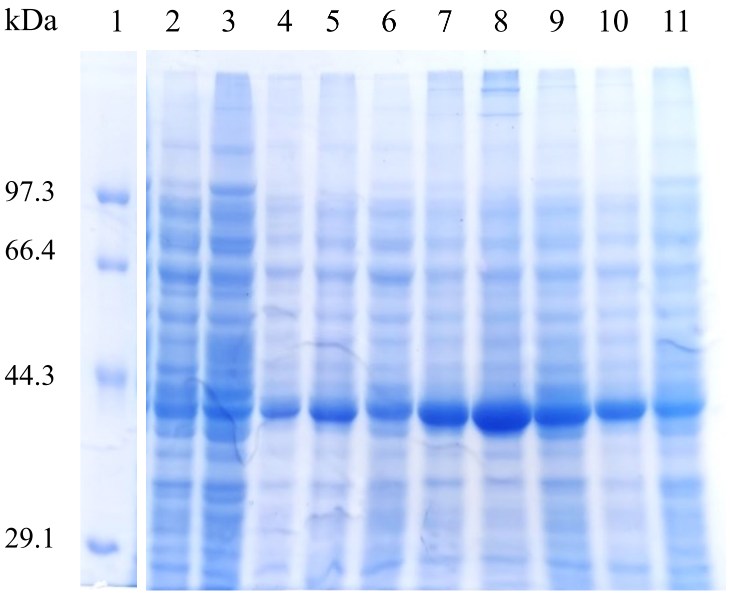
1、Marker 2、PyAly 3、K172D 4、K172Q
5、K172T 6、K172W 7、K172Y 8、R143A
9、R143M 10、R143W 11、R143Y

2 7.11 SDS-PAGE



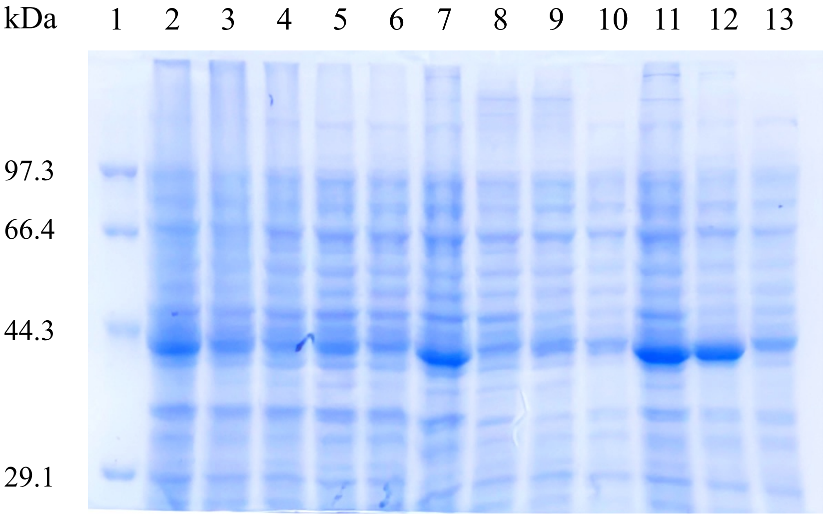
1.K172E 2. K172F 3. K172H 4. K172I
5. K172L 6. K172M 7. K172R 8.R143C
9. R143F 10. R143H 11. R143K 12. R143Q
13. R143T 14. R143V

3 8.8 SDS-PAGE (1)



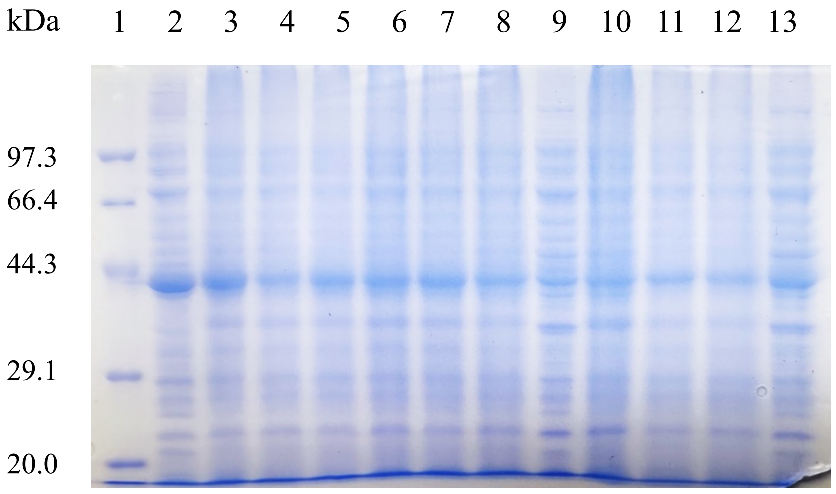
1、Marker 2、 K172A 3、 K172C 4、 K172S
5、 K172V 6、R143D 7、R143G 8、R143I
9、R143L 10、R143N 11、R143S

4 8.8 SDS-PAGE(2)



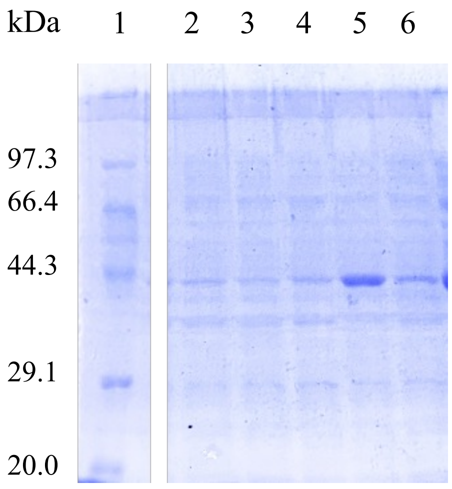
1、Marker 2、R159C 3、R159Y 4、R159V 5、R159Q
6、R159E 7、R159S 8、R159I 9、R159H 10、R159M
11、R159G 12、R159D 13、R159K

5 8.9 SDS-PAGE(2)



1、Marker 2、PyAly 3、F170E 4、F170R 5、F170D
6、F170I 7、F170A 8、R159N 9、R159P 10、R159L
11、R159A 12、R159F 13、R159T

6 9.12 SDS-PAGE (2)



1、Marker

2、R159S-K172P

3、R159K-K172P

4、R159N-K172L

5、R159D-K172E

6、R159N-K172T

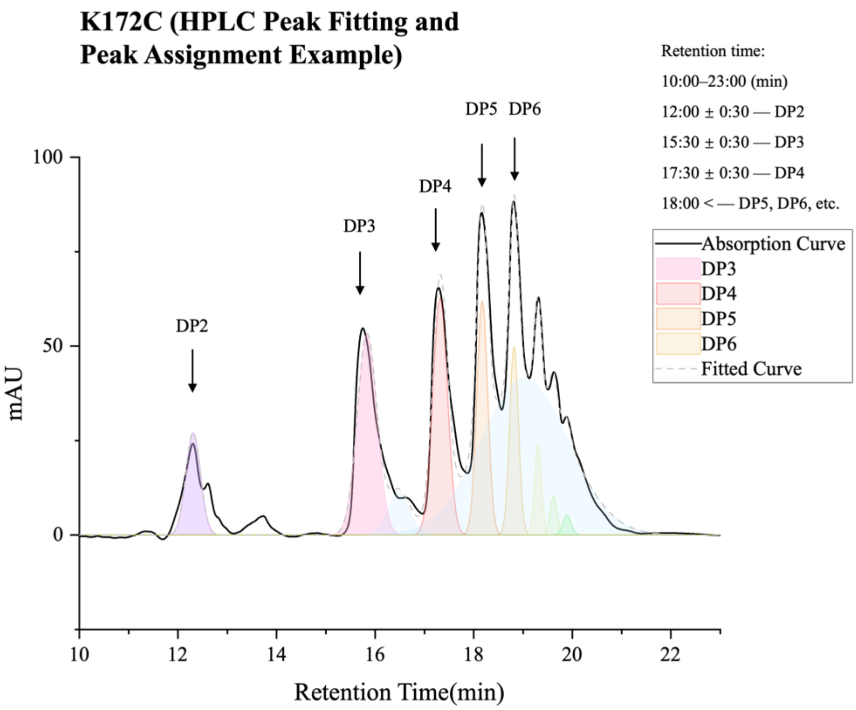
Supplementary Materials 3

High-Performance Liquid Chromatography (HPLC) Data

1 HPLC raw data link: https://doi.org/10.5281/zenodo.17238377

HPLC Data--- HPLC_Raw_Data .zip

2 HPLC peak fitting schematic： Retention time ranges and peak assignments are shown in the figure, with a schematic of peak fitting.



K172C (HPLC Peak Fitting and Peak Assignment Example)

Retention time:
10:00–23:00 (min)
12:00 ± 0:30 — DP2
15:30 ± 0:30 — DP3
17:30 ± 0:30 — DP4
18:00 < — DP5, DP6, etc.

3 Peak Areas and Relative Proportions of Mutant Products：

https://doi.org/10.5281/zenodo.17238377;

HPLC Data---Peak Areas and Relative Proportions of Mutant Products.xlsx

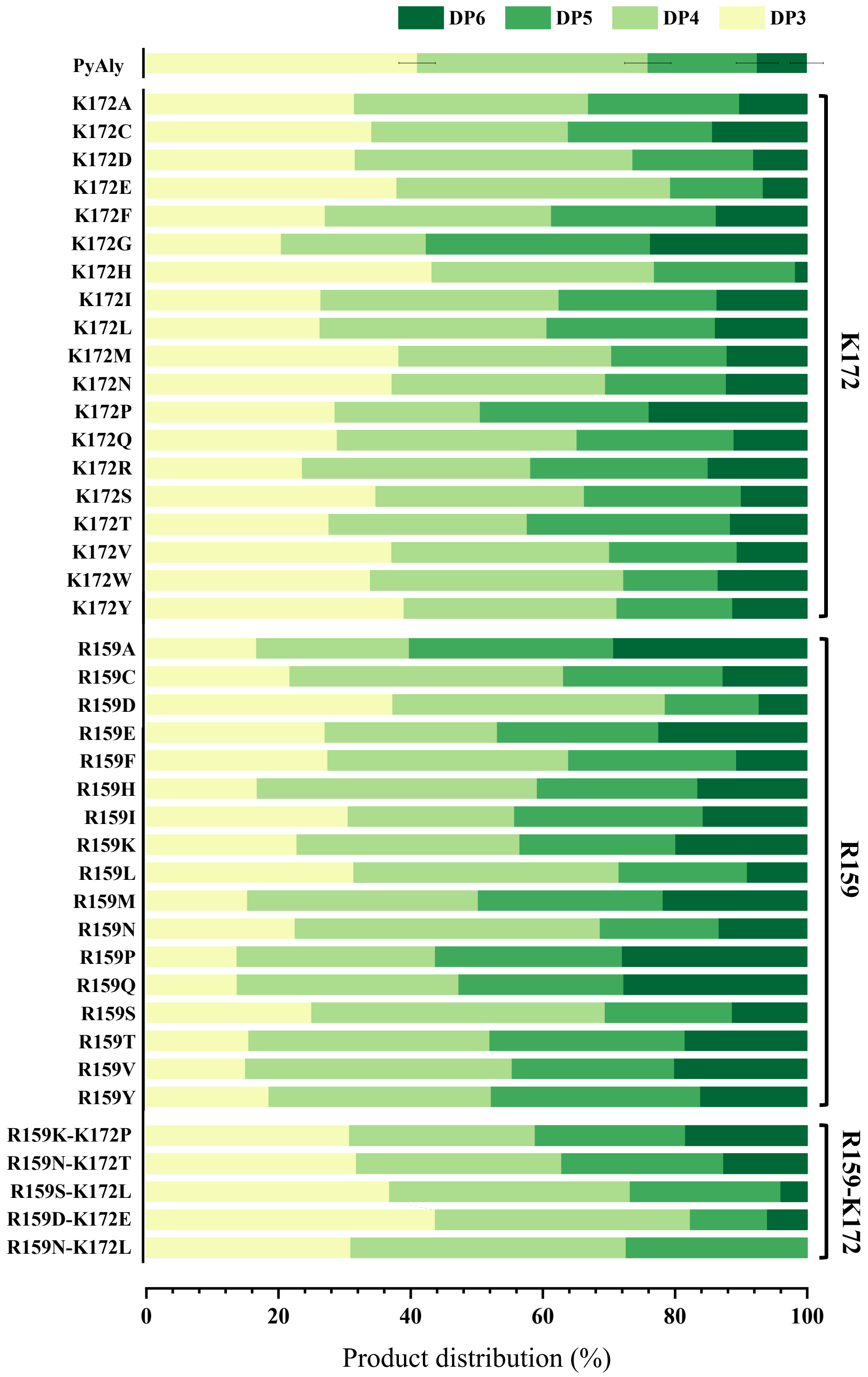
3.1 Table 1. Tetrasaccharide ratio and fold of K172 mutants

Mutant	Tetrasaccharide (%)	Fold vs. wild type
K172D	42.06	1.22
K172E	41.44	1.20
K172W	38.36	1.11
K172Q	36.30	1.05
K172I	36.07	1.04
K172A	35.44	1.03
K172R	34.58	1.00

3.2 Table 2. Tetrasaccharide ratio and fold of R159 mutants

Mutant	Tetrasaccharide (%)	Fold vs. wild type
R159N	46.20	1.34
R159S	44.46	1.29
R159H	42.41	1.23
R159C	41.44	1.20
R159D	41.24	1.19
R159V	40.39	1.17
R159L	40.18	1.16
R159T	36.53	1.06
R159F	36.47	1.06
R159M	34.96	1.01

4 Composite figure of all HPLC fitting data:



Supplementary Materials 4

Mechine Learning Data (Dry Experiment)

1 Training Dataset

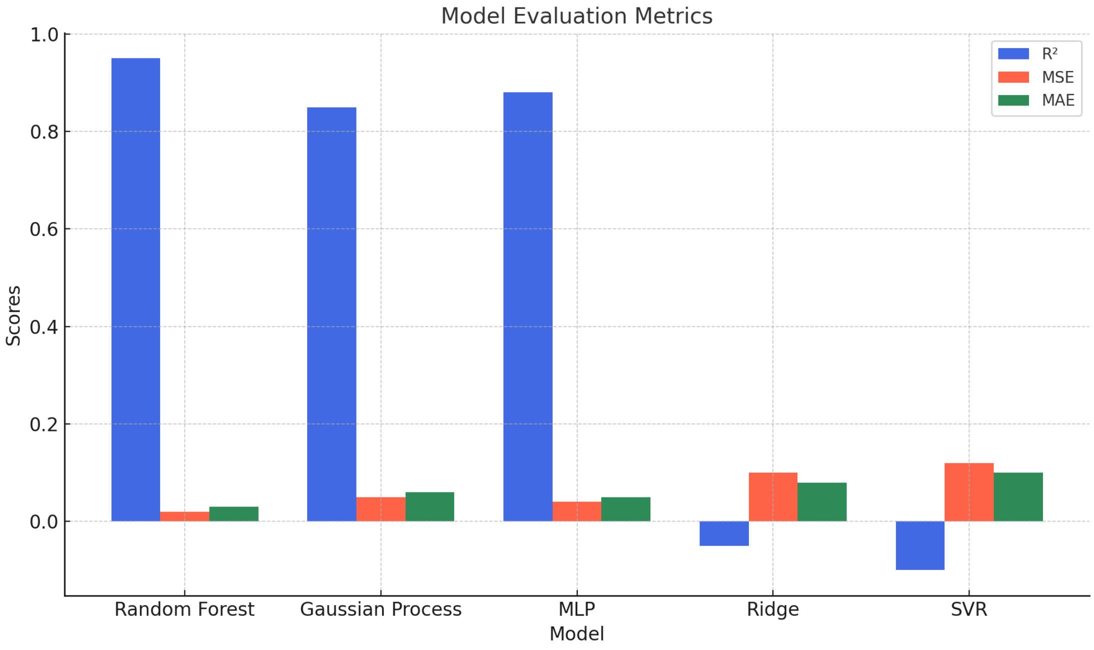
1.1 172_and_159_single_mutation_data.xlsx

variants	Ratio	Peak area	variants	Ratio	Peak area
PyAly	34.52	32.2825			
K172A	35.4381	25.8009	R159V	40.3924	12.9571
K172C	29.7699	23.3443	R159M	34.956	10.9895
K172D	42.0568	16.047	R159L	40.179	10.2779
K172E	41.4397	29.2107	R159A	23.1407	5.29627
K172F	34.2467	26.3641	R159I	25.2052	11.1866
K172G	21.9604	23.4536	R159D	41.245	13.8479
K172H	33.7035	24.6627	R159C	41.4444	34.123
K172I	36.0659	31.5488	R159E	26.0964	7.46749
K172L	34.3712	26.1933	R159F	36.4744	7.51798
K172M	32.2344	16.0669	R159H	42.413	15.7546
K172N	32.3268	30.6442	R159Y	33.6624	12.9394
K172P	21.9822	13.934	R159N	46.2035	23.8345
K172Q	36.3005	26.7457	R159K	33.7558	18.6557
K172R	34.5783	24.4967	R159T	36.5261	37.8827
K172S	31.6053	24.1437	R159Q	33.5588	10.6991
K172T	30.0067	29.7246	R159S	44.4598	33.6113
K172V	32.9917	28.8111	R159P	30.0349	9.09558
K172W	38.3581	21.6686			
K172Y	32.2159	17.6254			

1.2 159-172_double_mutation_data.xlsx

	PyAly	D-E	K-P	N-L	N-T	S-P
Ratio	34.52	38.60182	28.09219	41.65477	31.08149	36.41685
Peak area	32.28248	7.15491	25.16795	9.9071	24.85064	4.30634

2 Model Evaluation



R² (R-squared): Shows how well the model explains the variance in the data .

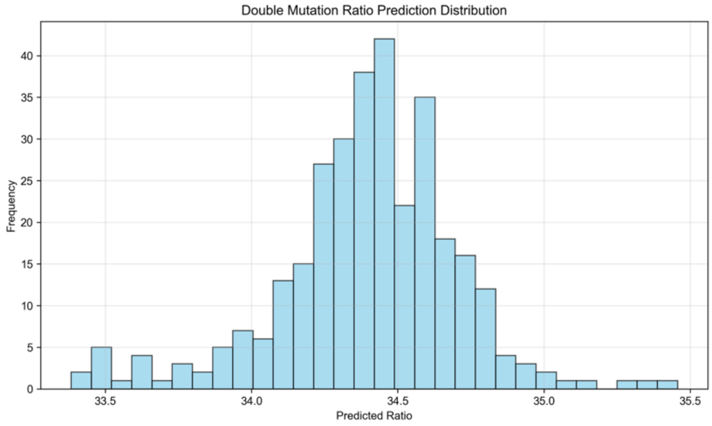
MSE (Mean Squared Error): Indicates the average squared difference between the predicted and actual values.

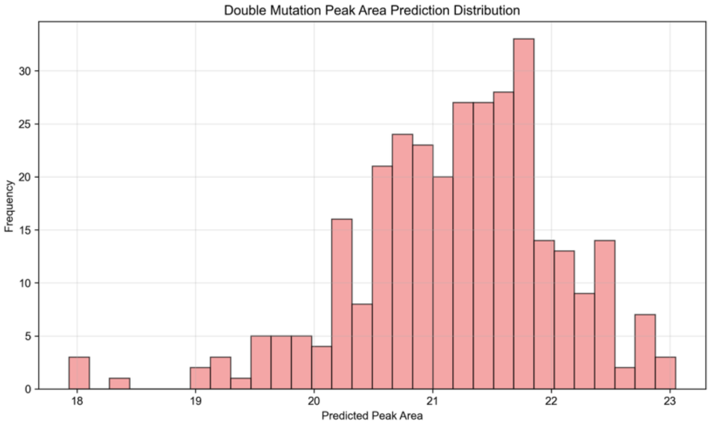
MAE (Mean Absolute Error): Represents the average absolute error.

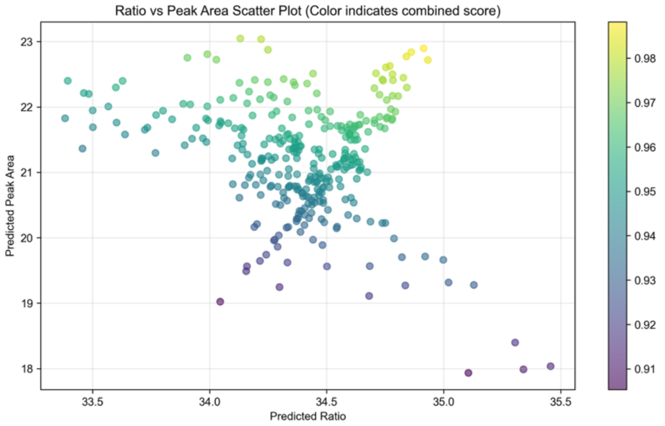
From the plot, we can see that the **Random Forest** model performs the best, with the highest R² and the lowest MSE and MAE, making it the ideal model for predicting mutation effects.

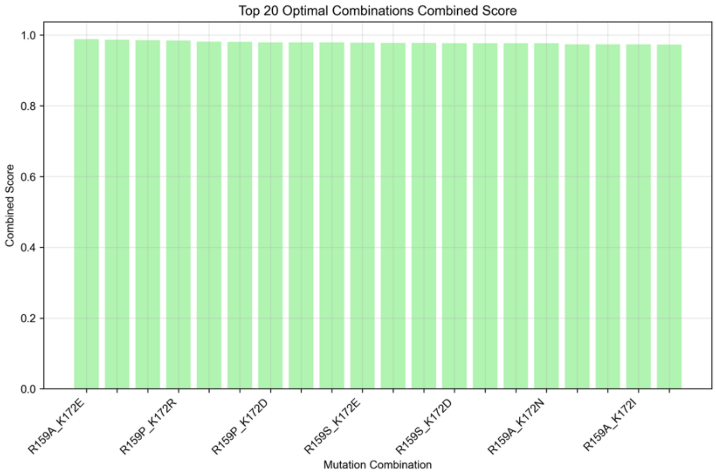
3 Visualization

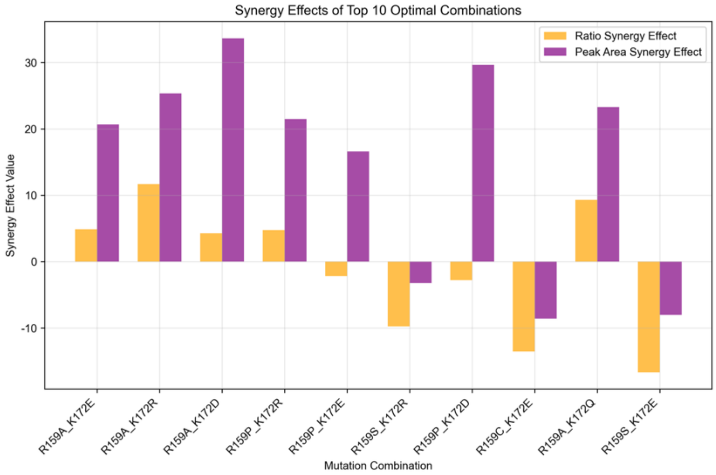
3.1 Mutation Predictions Analysis

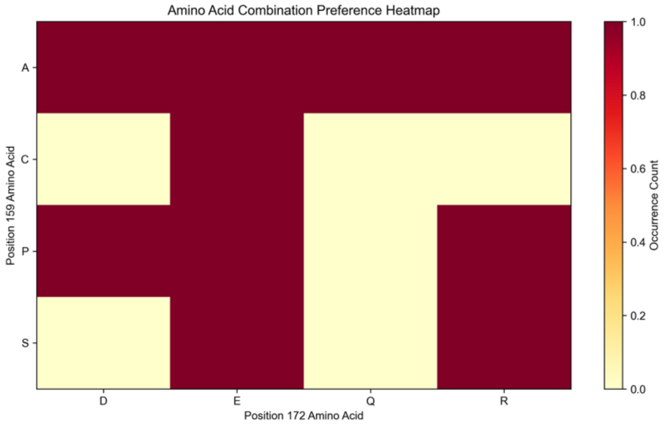




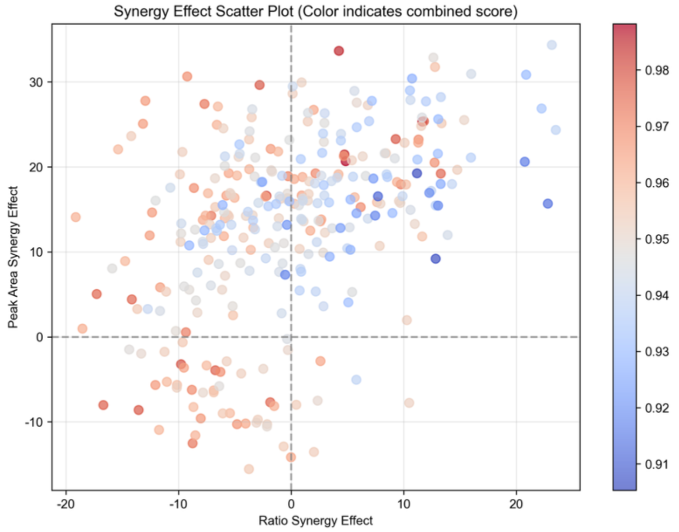




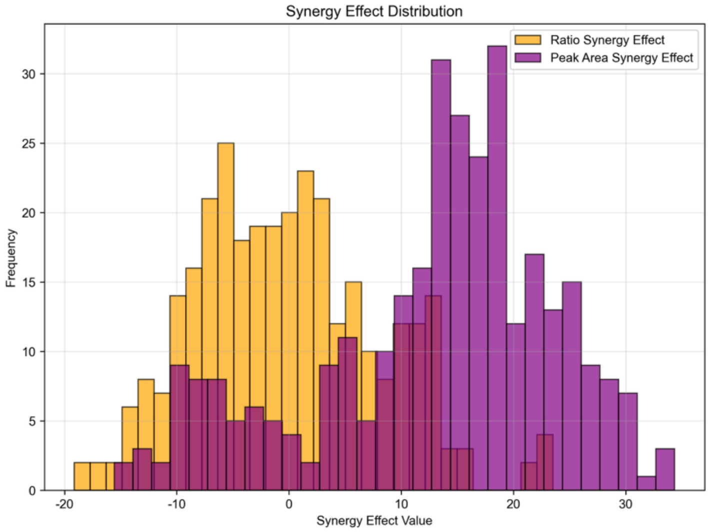




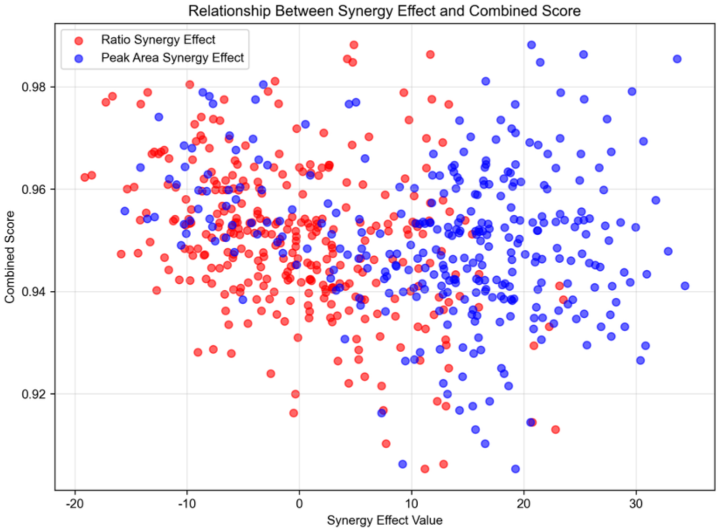
3.2 Synergy Analysis



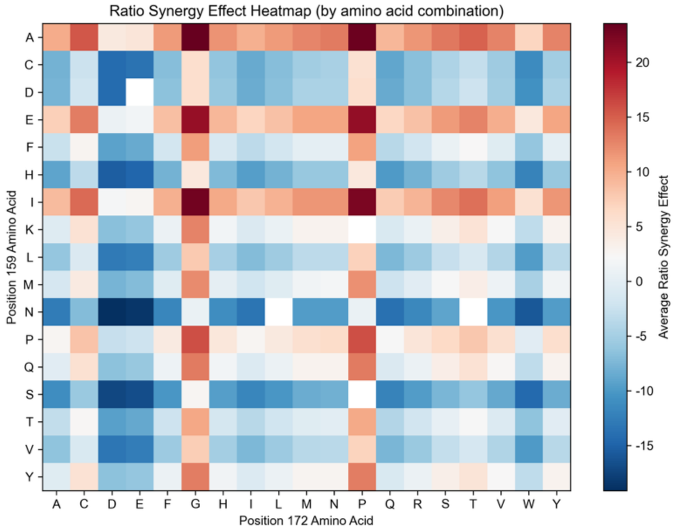
Synergy Effect Scatter Plot (Color indicates combined score)



Synergy Effect Distribution



Relationship Between Synergy Effect and Combined Score



Ratio Synergy Effect Heatmap (by amino acid combination)

3.3 318_Double_Putations_Predictions:

https://doi.org/10.5281/zenodo.17238377

Dry Experiment- all_318_double_mutations_predictions.xlsx

Supplementary Materials 5

Experimental record link:

https://vcnbl3t7yidp.feishu.cn/wiki/Uc1Xwi3GIiAsjDkr7L1cxobTnnc