Our experiments can be divided into 8 parts:

1. Created point mutation in E.coli

8.22

- We got the primers used in creating point mutation.

8.22-8.30

- According to the point mutation protocol, we did the following point mutation:

V34P	V488L
F438M	F438L
I410V	I410N
F413H	H415I
S437T	F413N
N493Y	V33I
D301R	N493L
R414Q	D301Y
F412Q	R414K
R36G	F412A
Y499W	R36E
S436G	Y499K
Y251F	S436A
L495M	Y251L
V33Y	L81S
V488W	I496V

9.1

- Prepared plasmid for sequencing.

9.5

- Transformed the correct plasmid into BL21.

9.6-9.10

- We used the transformed BL21 to do the whole cell catalysis.

9.15

- used the HPLC to see which bacteria with certain point mutation yield more acrylic acid.

2. Plasmids construction of saccharomyces cerevisiae

7.17

- Incubated Saccharomyces cerevisiae BY4741

7.19

- Transformed plasmid Y33 and Y22 into *E.coli* MG1655 according to plasmid transformation protocol.
- PCR

For plasmid Y33-URA-Ceas2（D301A）and Y33-URA-gld-DAK:

fragments	Forward primer	Reversed primer	Template	length（bp）
gldd	gld-d-F	gld-d-R	G.S	844
gldu	gld-u-F	gld-u-R	G.S	313
DAK	DAK-F	DAK-R	BL21-28a-DAK	1847
Ceas2-D301A	Ceas2-F	Ceas2-R	BL21-28a-Ceas2	1739
Y33c	Y33-c-F	Y33-c-R	Y33	6000
Y33gD	Y33gD-F	Y33gD-R	Y33	6500
PTDH3	PTDH3-F	PTDH3-R	Y33 or Y22	820
PGK1	PGK1-F	PGK1-R	Y22	801
pADH1	pADH1-F	pADH1-R	Y33	638

pcr system：200μl

Component	Concentration
sterile water	127μl
5×Fast pfu Buffer	40μl
dNTPs	16μl
Forward primer	5μl
Reversed primer	5μl
template	4μl
Fastpfu	3μl

Method:

	STEP	TEM	TIME	CYCLES
1	Initial	95℃	2min	1
2	denaturation	95℃	20s	
3	Annealing	58℃	20s	
4	extension	72℃	2-4kb/min	
5	circulation	Repeat the process2-4		34
6	extension	72℃	5min	1
7	Hold	4℃	Hold	

- Electrophoresis:

  Result: DAK、Ceas2、PTDH3、PGK1、pADH1 were correct

  Extracted DNA gel and measure the concentration of DNA.

7.23

- PCR: Y33gD and Y33c

  Result: Y33c was correct

7.24

- Gibson(according to the Gibson assembly protocol):

For Y33-URA-Ceas2（D301A）: 20ul

Gibson system	15ul
Y33c	4.65ul
PTDH3	0.25ul
Ceas2-D301A	0.25ul

- Transformed into the competent cell of BL21-DE3, plated (LB medium, Amp resistance).

7.25

- Transformed plasmid PCAS into *E.coli BL21* according to the plasmid transformation protocol.
- Pipetted 500ul of BL21-Ceas2 and BL21-Ceas2（D301A）into 800ml of 2YT medium.
- The Gibson assembly for Y33-URA-Ceas2（D301A）failed. We tried again.
- Activated BY4741: 5mlYPD + 20ul bacterial glycerol stocks of BY4741.

7.26

- Transformation of PCAS and the Gibson assembly for Y33-URA-Ceas2（D301A）succeeded. Pick bacteria on the plate and throw the tip into tubes.

7.27

- Creating bacterial glycerol stocks for long-term storage of the bacteria with the plasmid of Y33-URA-Ceas2（D301A）and PCAS respectively. (1ml bacteria + 500ul 50% glycerol).
- Colony PCR system: 20ul

  Dream Taq enzyme（mix）:10ul

  Forward primer(ceas2/PTDH3/Y33C): 0.5ul

Reversed primer(ceas2/PTDH3/Y33C):0.5ul

Template:0.5ul

H20:8.5ul

Plasmid preparation of the remaining 4ml mixture of bacteria and medium

- PCR for Ceas2, succeeded. Extract the gel and measure DNA concentration.
- Fusion(the first step):

Ceas2+PTDH3;PGK1+DAK;**Fast Pfu enzyme, 20ul**

fragments	Length(bp)	M(ng)(0.5pm)	C(ng/ul)	Template V(ul)
Ceas2	1739	565	106.3	5.32
PTDH3	820	266	274.7	0.97
PGK1	1847	600	396.5	1.514
DAK	801	260	331.6	0.785

Fast Pfu Buffer:4ul

dNTPs:1.6ul

template：showed above

Fast Pfu：0.3ul

H_2O:to make final volume as 20ul

7.28

- Fusion(the second step):Ceas2+PTDH3;PGK1+DAK;**Fast Pfu enzyme, 200ul**

fragment	Ceas2+PTDH3	PGK1+DAK
Forward primer	Ceas2-F	DAK-F
reversed primer	PTDH3-R	PGK1-R

Fast Pfu Buffer:40ul

dNTPs:16ul

Forward primer:5ul

reversed primer:5ul

template:20ul(18ul)

Fast Pfu:3ul

H_2O:112ul

Failed because we used wrong primers.

Decided the plasmid to construct:

plasmid	fragment	F	R	length	T
Y33-URA-gld-DAK	gld-Y33u-Y33d	gld-tGPD1-F	Y33gDd-U-R	3682	Y33
	Y33u-Y33D-DAK	Y33gDu-U-F	DAK-tPFK1-R	2808	Y33
	PGK1-ADH1-gld	PGK1-ADH1-F	gld-ADH1(R)	618	Y22
	ADH1-gld-tGPD1	ADH1-gld-F	tGPD-gld-R	1133	synthesize
Y33-Leu-Ceas2	pTDH3-Y33u-Leu	pTDH3-Y33u-F（Y33c-F）	Leu-Y33u-R	2375	Y33
	Y33u-Leu-Y33d	Y33u-Leu-F	Y33d-Leu-R	1631	Y181
	Leu-Y33D-Ceas2	Leu-Y33D-F	Ceas2-tPFK1-R（Y33C-R）	2578	Y33
	pTDH3+Ceas2			2539	
Y33-Leu-Ceas2-Nox	pTDH3-TEF2-Nox	pTDH3-TEF2-F	Nox-TEF2-R	583	p-T4（ALD6-ACC）
	TEF2-Nox-tRPS2	TEF2-Nox-F	tRPS2-Nox-R	638	synthesize
	Nox-tRPS2-Y33u	Nox-tRPS2-F	Y33Leu-tRPS2-R	320	p-T4（ALD6-ACC）
	tRPS2-Y33u-Leu	tRPS2-Y33Leu-F	Leu-Y33u-R	2375	Y33
Y181	tPFK1-Y181-pTDH3			5718	
	Y181-Y181u-tPFK1			2539	
	Ceas2-tPFK1-Y181			363	

PCR：Y33u-Leu-Y33d and TEF2-Nox-tRPS2

7.29

- Repeat fusion PCR(the second step) the same as yesterday.

  Result: no stripe

- PCR：Y33u-Leu-Y33d and TEF2-Nox-tRPS2
- Result: no stripe
- Activated BL21-Y33-URA-Ceas2, transformed into BY4741. Activated BY4741, incubated overnight.

7.30

- Transfered BY4741 into 50ml YPD medium and incubated 4-5h. Transformed Y33-URA-Ceas2（D301A）plasmid into BY4741.
- PCR：tPFK1-Y181-pTDH3、Y181-Y181u-tPFK1、Ceas2-tPFK1-Y181

  Extracted the gel and measured DNA concentration. The concentration of tPFK1-Y181-pTDH3 is low, PCR again.

- PCR：Leu-Y33D-Ceas2、pTDH3-Y33u-Leu、tRPS2-Y33u-Leu、Y33u-Y33D-DAK、gld-Y33u-Y33d、PGK1-ADH1-gld

  PGK1-ADH1-gld and tRPS2-Y33u-Leu were successful.

7.31

- Transformed Y181 plasmid into BL21-DE3
- PCR(changed Tm into 55℃):Leu-Y33D-Ceas2、pTDH3-Y33u-Leu、Y33u-Y33D-DAK、gld-Y33u-Y33d；
- PCR: tPFK1-Y181-pTDH3

  Fusion PCR of Gld but there is no stripe

8.1

- Purified PCR product from yesterday.

  Gel extraction: pTDH3-Y33u-Leu、Y33u-Y33D-DAK

- Fusion PCR of Gld
- Prepare bacterial glycerol stocks of BL21-Y181 and prepare plasmid Y181.
- PCR: Leu-Y33D-Ceas2、gld-Y33u-Y33d、tPFK1-Y181-pTDH3

8.2

- Transferred BY4741 into 5ml of YPD.
- Fusion PCR of Gld

  Forward: zbzf1-1

  Reversed: zbzf2-14
- Transformed plasmid carried T4 and T5 promoter into BL21-DE3.
- PCR: PTDH3-TEF2-NOX 、 NOX-TRPS2-Y33U 、 Leu-Y33D-Ceas2 、 gld-Y33u-Y33d、tPFK1-Y181-pTDH3

  Electrophoresis: PTDH3-TEF2-NOX and NOX-TRPS2-Y33U were correct.
- PCR: PTDH3-Y33U-Leu、Y33U-Leu-Y33d、Leu-Y33D-Ceas2

  Fusion PCR: Y33-Leu-Ceas2 and Y33-Leu-Ceas2-NOX(abbreviated as “PTDH3+Ceas2”)

  Electrophoresis: Y33-Leu-Ceas2 was correct.

  PCR: Y33-Leu-Ceas2-NOX

  Electrophoresis: correct.

  Concentration: 42ng/ul
- PCR:Leu-Y33D-Ceas2、gld-Y33u-Y33d、PTDH3-Y33U-Leu、Y33U-Leu-Y33d、Leu-Y33D-Ceas2

  Changed Tm of gld-Y33u-Y33d into 50℃.

  Electrophoresis: Y33U-Leu-Y33d was correct.

  Concentration: 36ng/ul

- Fusion PCR: Ceas2-tpFK1-Y181 和 Y181U（0.5pmol）

The first step:

fragment	length/bp	weight/ng	concentration/(ng/ul)	volume/ul
Y181U	2539	825.175	140.9	5.856
Ceas2-tpFK1-Y181	363	117.975	84.4	1.398

20ul system：

Fast Pfu Buffer: 4ul

dNTPs: 1.6ul

template: as showed above

Fast Pfu：0.3ul

Add H_2O to make final volume as 20ul.

The second step: Ceas2-tpFK1-Y181+Y181U

Fragment	Ceas2-tpFK1-Y181+Y181U
Forward	Y181-PTDH3 -F
Reversed	Y181-tpFK1-R

Result: no stripe

8.3

- Fusion PCR (step 1)system:**20ul Fast Pfu enayme**

target	fragment	Volume(ul)	Extension time
Y181U+Tpfk1	Y181U	5.86	2min10s
	Tpfk1	1.4	
ADH1+gld	ADH1	1.78	1min
	gld	2.25	
trps2+nox+tef2	trps2	0.39	1min
	nox	1.58	
	tef2	1.43	

System:

Fast Pfu Buffer:4ul

dNTPs:1.6ul

template: as showed above

Fast Pfu：0.3ul

Add H_2O to make final volume as 20ul.

- Fusion PCR (step 2):

Fragment	Forward primer	Reversed primer
Y181U+Tpfk1	Y181-PTDH3-F	Y181-TPFK1-R
ADH1+gld	PGK1-ADH1-F	TGPD-gld-R
trps2+nox+tef2	PTDH3-TEF2-F	TRPS2-NOX-R

Result: no stripe of Y181U+Tpfk1, correct stripe of ADH1+gld and trps2+nox+tef2

Transformed plasmid carried T4 promoter into BL21.

Did the fusion PCR (step 1) for Y181U+Tpfk1 again.

8.4

- Did the fusion PCR (step 2) for Y181U+Tpfk1 again. Succeeded.
- Gibson system:15ul
- Plasmid 1: Y33-URA-gld-DAK
- Plasmid 2: Y33-Leu-Ceas2
- Plasmid 3: Y33-Leu-Ceas2-Nox
- Plasmid 4: Y181-Leu-Ceas2

plasmid	fragment	volume/ul.
1.Y33-URA-gld-DAK	gld-Y33u-Y33d	0.9258
	Y33u-Y33D-DAK	1.5969
	ADH1+gld	0.7713
	PGK1+DAK	1.7060
2.Y33-Leu-Ceas2	pTDH3-Y33u-Leu	0.7539
	Y33u-Leu-Y33d	0.3161

	Leu-Y33D-Ceas2	3.6454
	pTDH3+Ceas2	0.2845
3.Y33-Leu-Ceas2-Nox	TEF2+Nox+ tRPS2	0.2747
	tRPS2-Y33u-Leu	0.3330
	pTDH3+Ceas2	0.2943
	Y33u-Leu-Y33d	0.3270
	Leu-Y33D-Ceas2	3.7710
4.Y181-Leu-Ceas2	tPFK1-Y181-pTDH3	3.7477
	Y181-Y181u-tPFK1	1.0110
	Ceas2-tPFK1-Y181	0.2413

- Redesign the primer because PCR for some fragments weren't successful.

  PCR with the new primer: tPFK1-Y181-pTDH3 and Leu-Y33D-Ceas2

8.5

- Gibson for plasmid 1 and 4 was successful.
- PCR Leu-Y33D-Ceas2 again.

  System:

  10*Fast Taq Buffer:20ul

  dNTPs:16ul

  F：5ul

  R：5ul

  template：4ul

  Fast Taq：2ul

  H_2O:148ul

  Fast Taq enzyme:

length	speed
0~2kb	10s/kb
2~3kb	20s/kb
>3kb	30s/kb

- Gibson:

2.Y33-Leu-Ceas2	pTDH3-Y33u-Leu	0.4815
	Y33u-Leu-Y33d	0.2034
	Leu-Y33D-Ceas2	4.1284
	pTDH3+Ceas2	0.1831
3.Y33-Leu-Ceas2-Nox	TEF2+Nox+ tRPS2	0.1746
	tRPS2-Y33u-Leu	0.2117
	pTDH3+Ceas2	0.1871
	Y33u-Leu-Y33d	0.2079
	Leu-Y33D-Ceas2	4.2188

8.6

- Transformed the plasmid 2 and 3 into BL21, plating.
- Transformed Y33-Leu-Ceas2（D301A）into BY4741.
- Colony PCR: bacteria with plasmid 1 and with plasmid 4 in them. Dream Taq enzyme system:20ul

Dream Taq Mix:10ul

F:0.5ul

R:0.5ul

Template:0.5ul

ddH_2O:8.5ul

result: prepared bacterial glycerol stocks of plasmid 4 and sent it for sequencing.

8.7

- The transformation of plasmid 2 and 3 failed.
- Electrophoresis: PGK1+DAK, Y33u-Y33D-DAK, tRPS2-Y33u-Leu and pTDH3+Ceas2 were not correct.

- PCR: Y33u-Y33D-DAK, tRPS2-Y33u-Leu

  Result: tRPS2-Y33u-Leu was correct.
- Fusion PCR: PGK1+DAK,pTDH3+Ceas2
- PCR: Y33u-Y33D-DAK(adjust Tm to 55℃)

  Failed again.
- Gibson: plasmid 2 and 3. Transformed the plasmid 2 and 3 into BL21, plating.

8.8

- PCR: Y33u-Y33D-DAK

  Result: succeed.
- Gibson: plasmid 1. Transformed the plasmid 1 into BL21, plating.
- Colony PCR: Y33-Leu-Ceas2(D301A)

  Result: succeed.

  Transferred Y33-Leu-Ceas2(D301A) into 5ml of CM-Leu.
- PCR: Y33u-Leu-Y33d

  Result: succeed.
- Fusion PCR: plasmid 3.

  TEF2+Nox+ tRPS2 and tRPS2-Y33u-Leu, Y33u-Leu-Y33d and Leu-Y33D-Ceas2

  Result: no stripe

  Did the fusion PCR again.
- Transferred BY4741 into 50ml of YPD medium(OD=0.2). When OD=0.8~0.9, transformed Y181-Leu-Ceas2 into BY4741, plating.
- Gibson: plasmid 3.

8.9

- The transformation of plasmid 2 succeeded. Activated the bacteria from the plate.
- Fusion PCR step 2 failed(0.1pm,Tm=55℃), change the Tm into 58℃,failed again.
- Gibson: plasmid 2 and 3(0.08pm)

8.10

- The transformation of plasmid 4 succeeded. Activated the bacteria from the plate.
- Transferred BY4741 into 50ml of YPD medium(OD=0.2). When OD=0.8~0.9, transformed each fragment of plasmid 1 and 2 (400ng) into BY4741, plating.

8.12

- The transformation of plasmid 1 failed, plasmid 2 succeeded. But colony PCR showed that the stripe was wrong.
- Gibson: plasmid 1 and 3(0.03pm)
- Fusion PCR: ADH1+gld、Leu+Y33D

  Result: PCR of ADH1+gld was successful.

8.13

- Transferred BY4741 into 50ml of YPD medium(OD=0.2). When OD=0.8~0.9, transformed each fragment of plasmid 1 and 3 (400ng) into BY4741, plating.
- One-step cloning and Gibson(0.03pm), transformed into BL21 and plated:

1．Y33-URA-gld-DAK	gld-Y33u-Y33d	0.281
	Y33u-Y33D-DAK	0.2882
	ADH1+gld	0.1288
	PGK1+DAK	0.277
3.Y33-Leu-Ceas2-Nox	TEF2+Nox+ tRPS2	0.208
	tRPS2-Y33u-Leu	0.155
	pTDH3+Ceas2	0.324
	Leu+ Y33D	2.0726
2.Y33-Leu-Ceas2	pTDH3-Y33u-Leu	0.4815
	Y33u-Leu-Y33d	0.2034
	Leu-Y33D-Ceas2	4.1284
	pTDH3+Ceas2	0.1831

8.14

- The transformation of plasmid 2 succeeded.
- PCR: Y33-URA-gld-DAK
- Fusion PCR: ADH1+gld and PGK1+DAK

  Result: succeeded.

8.15

- Sent plasmid 2 for sequencing.
- The transformation of plasmid 1 succeeded. Activated the bacteria ,prepared the plasmid and transformed the plasmid into Ecoli.
- PCR: ADH1+gld+PGK1+DAK, ADH1+gld.
- Gibson: plasmid 1.

1．Y33-URA-gld-DAK	Y33-URA-gld-DAK	2.936
	ADH1+gld	2.511
	PGK1+DAK	0.347

- Colony PCR of fragments of plasmid 3: pTDH3+Ceas2, TEF2+Nox+ tRPS2

  Result: no stripe
- PCR: Y33-Leu-Ceas2-Nox

System: 50ul, fusion enzyme.

5*RfHF buffer：10ul

10mM dNTPs:1ul

F: 2.5ul

R: 2.5ul

Template(Y33-Leu-Ceas2)：10ng（135.1ng/ul　0.074ul）

Enzyme: 0.5ul

Add H_2O to make final volume as 50ul.

Result: no stripe

8.16

- Transformation of plasmid 3, activated the bacteria for preparing plasmid.
- One-step cloning of plasmid 1.
- The sequence of plasmid 2 is correct. Transformed plasmid 2 into saccharomyces cerevisiae and activated BY4741. However, the bacteria didn't grow.

8.17

- Colony PCR: plasmid 1(ADH1+gld and PGK1+DAK)

  Result: falied.
- Activated BY4741, Transformed plasmid 2 into BY4741.
- One-step cloning: plasmid 1.
- Gibson: plasmid 1, plasmid 3.
- Prepared plasmid of plasmid 3.

8.18

- Transformed plasmid 3 into BL21-DE3.
- Activated BY4741 and transformed plasmid 1 into BY4741.
- One-step cloning: plasmid 1, plasmid 3. Transformed them respectively into Trans5-α, incubated overnight.
- PCR: TRPS2+NOX

  Result: succeeded.

8.19

- Plated Trans5-α incubated yesterday.
- Fusion PCR: PGK1+DAK
- Gibson: plasmid 1（Y33-URA-GLD-DAK）:

  System: 15ul

Fragment	Concentration(ng/ul)	length/bp	volume/ul
ADH1+GLD	71.6	1751	0.489
PGK1+DAK	62.9	2628	0.8356
Y33U	255.5	3682	0.2882
Y33D	190	2808	0.2956

Transformed plasmid 1 into BL21.

8.20

- Colony PCR:

  Plasmid 1: ADH1+TGPD1、PGK1+DAK

  Plasmid 3: TEF2+NOX+TRPS2

  Result: The transformation of plasmid 1 succeeded. Not sure about plasmid 3.

8.21

- Sent plasmid 1 and 3 for sequencing.

8.23

- PCR: plasmid 3, DAK
- Fusion PCR: PGK1+DAK
- Gibson: plasmid 1 and 3

plasmid	fragment	concentration(ng/ul)	length/bp	volume/ul
plasmid1	ADH1+GLD	71.6	1751	0.489
	PGK1+DAK	37.6	2628	1.40
	plasmid1 backbone	102	6444	0.49
	H_2O			1.85
plasmid3	TEF2+NOX+TRPS2	38.8	1500	0.77
	PTDH3+CEAS2	153	2539	0.33
	plasmid3 backbone	105.1	6534	1.24
	H_2O			2.66

Transformed the plasmid into Trans1-T1.

8.24

- Colony PCR: plasmid 1 and 3

  Result: closed to the correct stripe
- Activated the bacteria.

8.25

- Sent plasmid 3 for sequencing.

  Result: plasmid 1 missed fragment PGK1+DAK.

  Site mutation occurred in fragment NOX of Plasmid 3

9.4

- Activated Trans1-T1-Y33-Leu-Ceas2-NOX

9.5

- Activated BY4741
- Prepared plasmid 3 from the bacteria which had been activated yesterday.
- Transformed the plasmid into BY4741

9.6

- Activated the bacteria which had been transformed plasmid 3 into.
- Colony PCR: plasmid 3

  Result: correct. Activated the bacteria and prepared bacterial glycerol stocks.

9.7

- Fusion PCR: gld+DAK, ADH1+gld.

  Result: succeeded.
- PCR: backbone of plasmid 1.

  Template: Y33

F: gld-TGPD1-F

R: DAK-TPFK1-R

9.9

- PCR: PGK1+DAK, ADH1+gld, the backbone of plasmid 1.

9.10

- Colony PCR: S-gld-DAK
- Fusion PCR:

  ADH1+gld

fragment	concentration	length	volume	H2O
ADH1	246	618	1.6	13.6
gld	234	1113	0.3	

9.12

- PCR: the backbone of plasmid 1
- PCR: pGK1+DAK
- Gibson: S-gld-DAK, transformed into BL21.

9.13

- Colony PCR: S-gld-DAK
- Fusion PCR: pGK1+DAK

fragment	concentration	length	volume	H2O
pGK1	165	781	0.1	9.5
DAK	90.9	1827	0.4	

9.14

- Gibson: S-gld-DAK, transformed into BL21.

9.15

- Colony PCR: BL21-S-gld-DAK
- Transformation:
- Transformed Y33-LEU-ceaS2-NOX into BY4741△DLD1
- Transformed Y181-LEU-ceaS2 into BY4741-△DLD1△GPD1
- Transformed Y33-LEU-ceaS2 into BY4741-△DLD1△GPD1
- Transformed Y33-LEU-ceaS2-NOX into BY4741△DLD1△GPD1
- Transformed Y33-LEU-ceaS2 into BY4741
- Transformed Y33-LEU-ceaS2-NOX into BY4741
- Transformed Y33-LEU-ceaS2 and Y33-URA-gld-DAk into BY4741
- Transformed Y33-LEU-ceaS2-NOX and Y33-URA-gld-DAk into BY4741

9.16

- Prepared bacterial glycerol stocks and plasmid of BL21-S-gld-DAK.
- Sent S-gld-DAK for sequencing.

9.17

- Transformed Y33-LEU-ceaS2 and Y33-URA-gld-DAk into BY4741
- Transformed Y33-LEU-ceaS2-NOX and Y33-URA-gld-DAk into BY4741

9.18

- One-Step Cloning: S-gld-DAK
- PCR: ADH1+gld

9.19

- One-Step Cloning: S-gld-DAK

9.21

- Gibson: S-gld-DAK

9.22

- Fusion PCR: pGK1+DAK and ADH1+gld
- One-Step Cloning: S-gld-DAK

9.23

- Transformed the backbone of plasmid 1, pGK1+DAK and ADH1+gld into the following:

BY4741
BY4741△DLD1
BY4741-△DLD1△GPD1
BY4741-Y181-ceaS2
BY4741△DLD1- Y33-ceaS2
BY4741- Y33-ceaS2-NOX
BY4741△DLD1- Y181-ceaS2
BY4741- Y33-ceaS2

9.24

- Transformed the backbone of plasmid 1, pGK1+DAK and ADH1+gld into BY4741△DLD1- Y33-ceaS2-NOX.

9.26

- PCR: gld+DAK

9.27

- Activated BY4741-△DLD1△GPD1 from the plate.

9.28

- Colony PCR: gld+DAK and URA
- Transformed Y33-ceaS2-NOX into BY4741△DLD1-Y33-gld-DAK

10.14

- Extracted the genome of BY4741△DLD1△GPD1-Y33-ceaS2-Y33-gld-DAK, BY4741△DLD1△GPD1-Y33-ceaS2-Y33-gld-DAK, BY4741△GPD1-Y33-NOX-gld-DAK , BY4741△GPD1-Y181-ceaS2-gld-DAK.
- Extracted the genome of BY4741△DLD1-Y33-gld-DAK-Y33-ceaS2-NOX.

10.15

- PCR: ceas2D301NM306F, ceas2T80H, ceas2D301N, ceas2V33I
  Template: pET28a-ceas2D301NM306F,
  pET28a-ceas2T80H,pET28a-ceas2D301N, pET28a-ceas2V33I
  Forward primer: tPFK1-ceas2-F
  Reversed primer: pTDH3-ceas2-R
- PCR: Y33-LEU-NOX(8814bp)
  Template: Y33-Leu-ceas2-NOX
  Forward primer: ceas2-pTDH3-F
  Reversed primer: ceas2-tPFK1-R

10.16

- One-step cloning: Y33-Leu-ceas2D301NM306F-NOX, Y33-Leu-ceas2T80H-NOX, Y33-Leu-ceas2D301N-NOX, Y33-Leu-ceas2V33I-NOX
  Transformed the plasmids respectively into the competent cell of BL21.

10.17

- Transformation of Y33-Leu-ceas2D301NM306F-NOX failed. Activated BL21-Y33-Leu-ceas2T80H -NOX, BL21-Y33-Leu-ceas2D301N -NOX, BL21-Y33-Leu-ceas2V33I-NOX
  Colony PCR, electrophoresis showed the stripe were correct.
- PCR: Y33-LEU-NOX

- One-step cloning: Y33-Leu-ceas2D301NM306F-NOX
  Transformed the plasmid into the competent cell of BL21.
- Activated BY4741-Y33-ura-gld-DAK

10.18

- Colony PCR, electrophoresis showed the stripe of
  BL21-Y33-Leu-ceas2D301NM306F –NOX were correct.
- Transformed Y33-Leu-ceas2T80H-NOX, Y33-Leu-ceas2D301N-NOX,
  Y33-Leu-ceas2V33I-NOX into BY4741-Y33-ura-gld-DAK

10.19

- Transformed Y33-Leu-ceas2D301NM306F -NOX into
  BY4741-Y33-ura-gld-DAK

10.21

- We did the sole cell catalysis of BY4741-Y33-ura-gld-DAK-ceas2
  D301NM306F-NOX, BY4741-Y33-ura-gld-DAK-ceas2T80H -NOX,
  BY4741-Y33-ura-gld-DAK-ceas2D301N -NOX,
  BY4741-Y33-ura-gld-DAK-ceas2V33I –NOX

10.24

- Prepared bacterial glycerol stocks of BY4741-Y33-ura-gld-DAK-ceas2T80H
  -NOX, BY4741-Y33-ura-gld-DAK-ceas2D301N -NOX,
  BY4741-Y33-ura-gld-DAK-ceas2V33I –NOX

10.26

- Prepared bacterial glycerol stocks of BY4741-Y33-ura-gld-DAK-ceas2
  D301NM306F -NOX

3. Plasmid construction of E▪coli

8.30

- Colony PCR: ceas2-NOX-CAT-PETDuet，gld-DAK-pCDFDuet

Fragments (Length)	primers
NOX+M+CAT（2372bp）	PET-NIX-F
	PET-CAT-R
gld+M(1282)	CDF-gld-F
	DAK-M-R
DAK(1855)	M-DAK-F
	CDF-DAK-R

System:SUPER PCR MIX,20ul

Mix:10ul

F:0.5ul

R:0.5ul

T:1ul

ddH2O:8ul

Result: no stripe

9.1

- Gibson

Plasmid	Fragment	Concentration(mg/ul)	Length(bp)	Volume(ul)	H2O(ul)
gld-DAK-pCDFDuet	CDF	28.8	3499	2.43	1.35
	gld+M	46.2	1282	0.55	
	E．DAK	55.1	1855	0.67	
ceas2-NOX-CAT-PETDuet	PET	53.3	5137	1.93	1.60
	NOX+M	70.9	788	0.22	
	F12	33	1100	0.67	
	F3	18.8	550	0.58	

Transformed each fragment into 200ul of DH5α competent cell.

- Activated BL21-PET28a-ceas2 and MG1655-PET28a-ceas2

9.6

- Colony PCR: DH5Aα-gld-DAK-pCDFDuet and DH5Aα-ceas2-NOX-CAT-PETDuet

Fragments (Length)	primers
NOX+M（788bp）	PET-NOX-F
	CAT-PETM-R
CAT(1612)	PETM-CAT-F
	PET-CAT-R
gld+M(1282)	CDF-gld-F
	DAK-M-R
DAK(1855)	M-DAK-F
	CDF-DAK-R

9.7

- Sent E.coli NOX-CAT and E.coli GLD-DAK for sequencing.
- Colony PCR: GLD

  MG1655 Ceas2 GLD DAK

  BL21 Ceas2 GLD DAK

  BL21 Ceas2 GLD DAK

  MG1655 Ceas2 GLD DAK

  Primer: CDF-GLD-F M-GLD-R

9.9

- PCR: CAT F12 and F3(but the concentration is low) ,PGK1+DAK.
- Fusion PCR: CAT F12 and F3

9.10

- Colony PCR: GLD-M-DAK(3109)

  Result: Failed. PCR of the fragments again.
- Gibson: E.coli NOX-CAT
- PCR:GLD+M(1282) primers: CDF-GLD-F, DAK-M-R
- electrophoresis: E.coli GLD-DAK

  Result: GLD+M, DAK , PCD were all correct.

9.12

- Gibson: E.coli GLD-DAK

  DNA purification, transformed the plasmid into BL21

9.14

- Colony PCR: GLD-DAK

  Result: Failed.
- PCR: CAT F3

9.15

- Fusion PCR: CAT F12 and F3
- PCR:GLD and CAT F3

  Result: there was stripe of GLD but no stripe of CAT F3.

9.16

- Gibson: GLD+M , DAK, PCD ; GLD+M+DAK , PCD, DNA purification, transformed the plasmid into BL21

9.17

- Colony PCR: E.coli GLD-DAK

  Result: no stripe
- Colony PCR: E.coli NOX-CAT

Primer: PET-NOX-F PET-CAT-R

Result: no stripe

- Fusion PCR: NOX+M and CAT (2344bp)

9.18

- Prepare plasmid of E.coli-NOX-CAT 2, 3, 4, 7, 9, 15 ,used it as template to do the PCR.

  Primer: PET-NOX-F, CAT-PET-R , PETM-CAT, PET-CAT-R

  Sent E.coli-NOX-CAT 2 for sepuencing.

9.19

- Gibson : E,coli CAT-NOX , fragments: NOX+M, CAT, PET ; NOX+M+CAT, PET

  Result: the sequence missed CAT.

- Colony PCR: E.coli-GLD-DAK 1,2,9,11,13

  Result: E.coli-GLD-DAK 2 and 11 were correct. Activate the bacteria from the plate.

- One-step cloning: E.NOX-CAT

  fragments: NOX+M+CAT(2372), PET(5137)

9.20

- Prepare plasmids of E.GLD-DAK 2 and 11 for sequencing.
- Fusion PCR: NOX+M, CAT(2344)

  Result: no stripe

- Colony PCR: E.NOX-CAT

  Result: the stripe of NOX existed but failed to see the stripe of CAT.

9.21

- Prepared plasmids of E-NOX-CAT.
- PCR:E-NOX-CAT. Primer: PET-NOX-F, CAT-PETM-R ; PETM-CAT,

PETM-CAT-R

- Gibson: E-NOX-CAT

  fragments: NOX+M ,CAT, PET.

- Sent E-GLD-DAK for sequencing.

  Primers: CDF-GLD-F, DAK-M-R; M-DAK-F,CDF-DAK-R

9.22

- One-step cloning: E.NOX-CAT

  fragments: NOX+M+CAT(2372), PET(5137)

- PCR: PET.

  Result: successful.

9.23

- Colony PCR: E,NOX-CAT

  Result: 4 and 15 were correct. Activated the bacteria from the plate.

- One-step cloning: GLD+M , E.DAK , PCD ; GLD+M+E.DAK, PCD

9.24

- Colony PCR: E,NOX-CAT and E.GLD-DAK
- Sent CAT for sequencing.
- Fusion PCR:CAT F1 and F2.
- PCR: F3
- Fusion PCR: F1+F2, F3
- One-step cloning: NOX+M+PET, CAT1 ; NOX+M+PET , CAT2;

  NOX+M+PET ,CAT3

- Colony PCR: E.GLD-DAK

  E.GLD-DAK 2 was correct. Activated the bacteria from the plate.

9.25

- Fusion PCR of F1+F2,F3 failed.

- PCR of NOX+M+PET failed.
- Activated bacteria of E.NOX-CAT from the plate.
- One step cloning and Gibson: F12, F3, NOX+M+PET
- Colony PCR: E.NOX-CAT

  Result: there was stripe of CAT but no stripe of NOX.

  Activated bacteria of 18,33,36.

9.26

- Sent E.coli NOX-CAT for sequencing. Primers: PET-NOX-F, NOX-PETM-F, PET-CAT-R

9.27

- The sequence of E.GLD-DAK is correct. We successfully constructed the plasmid of E.GLD-DAK.
- Fusion PCR of F1+F2,F3 failed.

9.28-9.30

- Still working on the fusion PCR of CAT F1,F2,F3 but failed.

10.1

- PCR: NOX+M+PET ,CAT
- One step cloning and Gibson: CAT, NOX+M+PET

10.2

- Colony PCR: NOX+M+CAT primers: PET-NOX-F, PET-CAT-R

  Result: 13 and 14 were close to the correct band marker but we weren’t sure.

  Activated bacteria of 13 and 14.
- Fusion PCR: CAT F1 and F2

10.3

- Prepare plasmid of E.NOX-CAT
- Fusion PCR: F12, F3 ;F1, F23

10.7

- Sent F1 for sequencing.

10.8

- The sequence of E.NOX-CAT was wrong.
- Prepared competent cells of BL21-Gld-Dak and MG1655
- Transformed plasmid Gld-DAK into MG1655

10.9-10.13

- Constructed CAT-f1 using CPEC method, the sequence was correct.

10.15-10.18

- Constructed CAT-f1,CAT-f2 and CAT-f3 using CPEC method and finally constructed CAT, the sequence was correct.

10.18-10.22

- One-Step Cloning: E.NOX-CAT

  The sequence was correct.

10.24

- Transformed plasmid E.NOX-CAT into competent cells of MCGD and ECGD.

  But there was no colony grew on the plate.

4. A test of the tolerance in acrylic acid of E ▪coli

7.20

- Activated bacterial glycerol stocks of BL21-DE3 in 5ml LB medium.

7.21

- 7:00 transferred the bacteria into 800ml LB medium
- 12:40 OD=1.14, dispensed the bacteria into 14 bottles, each bottle contains 50ml.

  Bottle No.1, 2: did not add acrylic acid

  No.3-5: add acrylic acid(final concentration of acrylic acid was 100mg/l)

  No.6-8: add acrylic acid(final concentration of acrylic acid was 500mg/l)

  No.9-11: add acrylic acid(final concentration of acrylic acid was 1000mg/l)

  No.12-14: add acrylic acid(final concentration of acrylic acid was 2000mg/l)

  14:40 Diluted 4 times, measured OD

  17:35 Diluted 10 times, measured OD

  19:40 Diluted 10 times, measured OD

  21:40 Diluted 10 times, measured OD

7.22

- 10:00 Diluted 20 times, measured OD

5. A test of the tolerance in acrylic acid of S. cerevisiae(BY4741)

7.19

- 9:00 Activated BY4741.
- 21:00 transferred BY4741 into 800ml of YPD.

7.20

- 9:30 Diluted 5 times, measured OD, OD=0.832.

dispensed BY4741 into 14 bottles, each bottle contains 50ml.

Bottle No.1, 2: did not add acrylic acid

No.3-5: add acrylic acid(final concentration of acrylic acid was 100mg/l)

No.6-8: add acrylic acid(final concentration of acrylic acid was 500mg/l)

No.9-11: add acrylic acid(final concentration of acrylic acid was 1000mg/l)

No.12-14: add acrylic acid(final concentration of acrylic acid was 2000mg/l)

11:30 Diluted 20 times, measured OD

13:30 Diluted 20 times, measured OD

15:30 Diluted 20 times, measured OD

7.21

- 9:40 Diluted 20 times, measured OD

6. gene knock out of saccharomyces cerevisiae

We planned to knock out 4 genes in saccharomyces cerevisiae: GPD1, DLD1, PDC1, PDC5.

8.15

- PCR: the upstream and downstream 1000bp of the 4 genes (with the genome of BY4741 as template)
- Result: there were stripe of PDC1 and PDC5 respectively, but the concentration both is low.
- PCR: the upstream and downstream 1000bp of DLD1 and GPD1.
- Result: no stripe
- PCR: pCAS_Phe-URA3 with 20bp homologous arm, DNA purification, DMT

enzyme digestion, transformed into BL21-trans5α, plated.

8.16

- Extract the genome of BY4741.
- Activated BL21-trans5α from the plate.
- PCR: the upstream and downstream 1000bp of the 4 genes (with the genome of BY4741 as template)
- Result: all were successful except the downstream of GPD1.
- Fusion PCR: the upstream and downstream of PDC1; the upstream and downstream of DLD1.

gene	fragment	length/bp	concentraion/(ng/ul)	volume/ul
PDC1	PDC1-U	1000	52.8	1.231
	PDC1-D	1000	69.6	0.934
DLD1	DLD1-U	1000	67.9	0.957
	DLD1-D	1000	45.9	1.416

System(20ul):

Pfu buffer: 4ul

dNTPs:1.6ul

T: as showed above

Pfu: 3ul

Add H2O to make final volume as 20ul

8.17

- Sent pCAS_Phe-URA3 for sequencing.
- PCR: the downstream 1000bp of GPD1.
- Fusion PCR: the upstream and downstream of PDC5

gene	fragment	length/bp	concentraion/(ng/ul)	volume/ul
PDC5	PDC5-U	1000	162	0.4012
	PDC5-D	1000	100	0.65

Transformed the fragments of PDC1, DLD1 respectively and the pCAS_Phe-URA3 into BY4741, incubated at 30℃.

8.18

- Fusion PCR: GPD1
- Transformed the fragments of PDC1, DLD1, PDC5, GPD1, PDC1+PDC5, DLD1+GPD1, PDC1+PDC5+DLD1+GPD1 respectively and the pCAS_Phe-URA3 into BY4741, incubated at 30℃.

8.19

- We discovered that knock out PDC1 and PDC5 affected the growth of the yeast. So we decided not to knock out the genes of PDC1 and PDC5. And the GPD1 was hard to knock out, we haven't knocked out the gene yet.
- As a result, we successfully knocked out DLD1

7. Whole cell catalysis of E.coli

Our team had two groups of people to do this part of experiment respectively.

Group 1:

7.25

- We got the bacterial glycerol stocks with following site mutation:
- D113A,D301A,D301A+R303A,D301H,D301H+R303A,D301N,D301N+M306F,D301N+M306F,D301N+M306N,E110K,E110P,E300A,E37A,H120F,H415R,I410M,M306F,M306N,R303A,R36A,R414A,R414V,R414Y,S305K,S305N+M306F,S305N+M306N,S305R,S305R+D301N,S305R+M366N,S436A,S436T,SRMF,T289N,T80H,V121E,Y298A,Y499A

7.31-8.10, 8.16-8.23

- Activated the bacterial glycerol stocks and did the whole cell catalysis according to the protocol.

9.1

- Used HPLC to see which kind of bacteria yield more acrylic acid

Group 2:

7.22

- Activated BL21-PET28a-Ceas2 and BL21-PET28a-Ceas2-D301A

7.23

- 14:00 transferred the bacteria into 300ml LB medium, incubated in 37℃, 220rpm/min.
- 16:40 OD=0.622, added 150ul of 1mol/L IPTG, incubated in 37℃, 220rpm/min for 14 hours.

7.24

- 9:00 conditions:
- pH:7.4 and 8.0
- substrate: 2% glucose and 2% glycerol
- bacteria: BL21-PET28a-Ceas2 and BL21-PET28a-Ceas2-D301A
- parallel: 3
- time: 4h, 8h,16h, 32h

8.19 – 8.22

- we discovered that the bacteria with the following site mutation yield more acrylic acid: S305R, D301N, I410M, M306N, R303A, E110P.
- Activated the bacterial glycerol stocks and did the whole cell catalysis according to the protocol.

9.30-10.7

- We did the whole cell catalysis of the following E.coli:
- MG-1655-Ceas2-Gld-DAK(MCGD),BL21-Ceas2-Gld-DAK(BCGD),BL21-Ceas 2(BC)
- BL21-Ceas2(BC),BL21-Ceas2-gld-DAK-NOX-CAT(BCGDNC)
- MG1655-Ceas2(MC),MG1655-Ceas2-gld-DAK-NOX-CAT(MCGDNC)
- Time: 21h, 42h

10.18-10.21

- We did the whole cell catalysis with the following site mutation: D301N+M306F, T80H, D301N, V33I, F413N, V33I, F412Q, Y251F
- Time: 21h, 42h

10.22

- We did the whole cell catalysis of the following E.coli:

1.BL-GD-NC-V33I

2.BL-GD-D301N+M306F

3.BL-GD-NC-T80H

4.BL-GD-V33I

5.BL-GD-NC-D301N

6.BL-GD-D301N

7.BC

8.BL-GD-NC-D301N-M306F

(BL: BL21, GD: Gld-DAK, NC: NOX-CAT, BC:BL21-Ceas2)

- Time: 42h

10.23

- We did the whole cell catalysis with the following site mutation: I410V, N493L, S436A
- Time: 21h, 42h

8. Whole cell catalysis of S. cerevisiae

8.13

- We incubated BY4741-Y33-URA-Ceas2-D301A.
- 21:00 OD=0.688*4
- Conditions:
- pH: 4.4 and 6.6
- substrate: glucose and glycerol(1% and 2% respectively)
- parallel repeat: 3
- reaction time: 24h(one day), 48h(two days), 96h(three days), 192h(four days), 384h(five days)
- pipet 1ml of the sample every 24h.

8.21

- We did the stress condition experiment of S. cerevisiae(BY4741, BY4741-Y33-Leu-Ceas2, BY4741-Y181-Leu-Ceas2) in order to see if we incubated the yeast in bigger space, they will yield more acrylic acid.
- Conditions:
- pH: 4.4 and 6.6
- substrate: glucose and glycerol(1% and 2% respectively)
- parallel repeat: 3
- reaction time: 24h(one day), 48h(two days), 96h(three days)

8.22

- We did the whole cell catalysis of BY4741-Y33-Leu-Ceas2, BY4741-Y181-Leu-Ceas2.
- pH: 4.5, 7.4 and 8.0
- substrate: 2%glucose and 2%glycerol
- parallel repeat: 3

- reaction time: 17h, 25h, 36h

9.10 – 9.15

- 19:20 we did the whole cell catalysis of BY4741(50ml) and BY4741-Y33-Leu-Ceas2(300ml).
- We did the stress condition experiment of BY4741(200ml), BY4741-Y33-Leu-Ceas2(400ml), BY4741-Y181-Leu-Ceas2(400ml).

9.30

- we did the whole cell catalysis of:
    1. BY4741-△DLD1-Y33-Ceas2-Y33-gld-DAK
    2. BY4741-Y33-Ceas2-Nox-Y33-gld-DAK
    3. BY4741-△DLD1-Y181-Ceas2-Y33-gld-DAK
    4. BY4741-Y33-Ceas2
- Conditions:
- pH: 4.5, 7.4 and 8.0
- substrate: 2%glucose and 2%glycerol
- parallel repeat: 3
- reaction time: 24h(one day), 48h(two days), 96h(three days)

10.3

- we did the whole cell catalysis of:

5.BY4741-Y33-Ceas2 -Y33-gld-DAK

6.BY4741 -Y181-Ceas2-Y33-gld-DAK

7.BY4741-Y33-Ceas2

8.BY4741-Y33-Ceas2

9.BY4741-△DLD1-Y33-Ceas2-Nox-Y33-gld-DAK；

- With the same conditions of 9.30

10.24

- we did the whole cell catalysis of:

1.BY4741-△DLD1-Y181-Ceas2-Y33-gld-DAK

2.BY4741-Y33-Ceas2-Nox-Y33-gld-DAK

3.BY4741-△DLD1-Y33-Ceas2-Y33-gld-DAK

4.BY4741-Y33-Ceas2 -Y33-gld-DAK

5.BY4741-Y33-GD-NOX-Ceas2-T80H

6.BY4741-Y33-GD-NOX-Ceas2-V33I

7.BY4741-Y33-GD-NOX-Ceas2-D301N

- Conditions:
- pH: 7.4 and 8.0
- substrate: 2%glycerol
- parallel repeat: 3
- reaction time: 96h(three days), 192h(four days)