NOTEBOOK

July 2023

JULY

HUBU-SKY-China

AUTHOR: Jiapeng Ruan

PCR, Prepare TB and Na2HP04 solutions

Sunday, July 1, 2023

Overview

- 28a, cl2 and adh for PCR and glue, 28a without strip, not P, re-run strip is not obvious
- Prepare TB medium, TB buffe, and 4X phosphate solutions in preparation for subsequent experiments and autoclave

Experimental equipment

- Sterile water, template, positive primer, reverse primer, primer star enzyme, PCR machine
- 1xTAE, agarose, microwave oven, loading, marker, nucleic acid electrophoresis instrument

Experimental steps

①PCR

- Add 1ul template (28A, Cl2, AdH), 3ul sterile water, 0.5ul positive primer, 0.5ul reverse primer, 5ul primer and 5ul primer star enzyme to the PCR tube
- Set the parameters of PCR instruments: predenaturation 95°C for 3min, denaturation 95°C 2Os, renaturation 66°C 2Os, extension 72°C for 1min, compound extension 72°C for 5min, protection instrument 12°C for 2min

②Gel electrophoresis

- Configure 0.8% nucleic acid glue: add 30 ml of 1xTAE, 0.24 g agarose to the

Erlenmeyer flask and dissolve in the microwave

- Nucleic acid glue dispensing: 2ul loading + 1 ul sample mixed, transferred to pores, left and right added 1Kb and 2000bp marker 2ul control
- nucleic acid electrophoresis: 150V constant pressure electrophoresis for about 20min, dark staining for 20min, rinsed with clean water and observed under the imager

③Configure TB culture medium

- 60.0g protein vein, 120.0g yeast powder, 20ml glycerin, deionized water
- Add Na2HP04 71.6 g to prepare 1L with deionized water and filter

2A De-template and T5 conversion

Sunday, July 2, 2023

Overview

- The 28a vector, Cl2 fragment, and ADH fragment after PCR were detemplated, and then the three fragments were ligated by T5 enzyme and transferred to DH5a competent cells

Experimental equipment

- DPN1 enzyme
- rcutsmart
- 4buffer
- T5 enzyme diluted but not added with 4buffer

Experimental steps

①De-template

- First, dilute DPN1 and add 1ul DPN1 enzyme, 1ul rcutsmart buffer, and 8ul sterile water to a 1.5ml EP tube.
- The diluted DPN1 enzyme was added to 2ul, 1ul rcutsmart buffer, 2ul sterile water, and 5ulPCR product to the anti-template system.
- Put it into a PCR machine and set the parameters: 37°C for 50min, 80°C for 20min, 12°C for 2min

②T5 conversion

- Add 1.5ulCL2 fragment, 1.5ulDH fragment, and 1.0ul28a vector to the 1.5 ml EP tube

- Add 0.5 ul of diluted T5 enzyme and 0.5ul4buffer to the EP tube above in the ultra-clean bench, and immediately ice bath for 5 min
- Add competent cells to the T5 system for 70ul in an ultra-clean bench and ice bath for 30 min
- Heat excitation: water bath in a 42°C water bath for 45s, and then immediately ice bath for 2min.
- Add NZY medium 150 ul to the ultra-clean bench
- Incubate at constant temperature for 40min-60min in a shaker at 37 °C
- . After incubation, 200ul of the bacteria were coated in LK medium plates and incubated overnight at 37°C

Pick bacteria, prepare protein purification

Sunday, July 3, 2023

Overview

- After T5 transformation, a single colony coated on the medium was selected, the shake flask effect was not good, and the time was not enough, so the PCR verification of the bacterial solution was not done
- Clean the purification column in preparation for tomorrow's purification

Experimental equipment

- Pipette gun, tip of pipette, ultra-clean bench, alcohol bottle, alcohol lamp, 1.SmlEP tube, LK medium
- RO water, 0.1MNaOH, purification column

Experimental steps

①Pick fungi

- Add 800ul LK medium to 1.5 ml EP tubes in the ultra-clean bench
- Carefully pick up the single colony with the tip and transfer into the EP tube
- Incubate at 37 °C shaker for 5 h

②Prepare protein purification

- Wash with RO water to dress the master
- Soak the purification column with 0.1MNaOH for 15 min before tipping away
- Soak in RO water

Ruptured bacteria, purification and transformation

Wednesday, July 5, 2023

Overview

- The bacteria containing the M8 and 8F genes are broken up to allow the corresponding proteins expressed to flow out of the cell
- The 28a-CL2-ADH plasmid was cloned and converted to increase its number

Experimental equipment

- Ruptured bacteria: high-pressure bacterial crusher, PBSbuffer, pmfs
- Protein purification: nickel beads, PBSbuffer, pmfs, imidazole, G250 Coomassie brilliant blue
- Transformation: competent E. coli, NZY medium, LK medium

Experimental steps

①Rupture bacteria

- Weigh the bacteria on the balance and add 10 ml of bacteria to resuspend
- Before sterilization, add 1% protease inhibitors PMSF30Ul and 35ul to the resuspended bacterial solution
- Wash the high-pressure cell disruptor twice with RO water and twice with PBS buffer, maintaining the pressure at 300bar-400bar during cleaning
- Add bacteria to the instrument, and the sterilization pressure is maintained at 900bar-1100bar
- Repeat the sterilization 3-6 times so that the bacterial solution is no longer viscous
- After the sterilization is completed, aliquot with 2ml EP tubes and centrifuge in a

centrifuge at 14000rpm 10°C for 30min (leave one tube without centrifugation for sample preparation)

- After centrifugation, one tube of pellet and one tube of supernatant (sample preparation) are retained. The rest can be collected in the supernatant.

②purification

- Start by adding an appropriate amount of nickel beads to the purification column and then wash twice separately with RO water and PBSbuffer.
- After cleaning, an appropriate amount of 10mM imidazole was added to the purified beads with nickel beads and soaked for 5min to activate the nickel beads.
- After activating the nickel beads, immediately add the collected upper clearing night, and close the tube mouth with parafilm and mix in a silent mixer for 60 min (so that the protein of interest fully binds to the nickel beads).
- After mixing, take the flow penetration solution and place it in ice.
- Elution was performed with 10 mM imidazole, 30 mM imidazole, 50 mM imidazole, 100 mM imidazole, 150 mM imidazole, 300 mM imidazole, and 600 mM imidazole. Each concentration of imidazole should be washed until the eluate encounters G250 Coomassie bright blue and does not change blue.

③transformation

- Add competent cells 25 ul and plasmid 1.5 ul to a 1.5 ml EP tube in an ultra-clean bench (plasmids can be added outside the table)
- Heat excitation: Water bath in a 42°C water bath for 45s, and then immediately ice bath for 2min

- Add NZY medium 150 ul to the ultra-clean bench
- Incubate at constant temperature for 40min-60min in a shaker at 37 °C
- After incubation, 100ul of bacterial solution was coated in LK resistant plates and reared overnight at 37°C

Pick bacteria, ultrafiltrate, draw plasmid & measure concentration & run nucleic acid glue

Thursday, July 6, 2023

Overview

- Plate singled colonies with the 28a-cl2-ADH gene were placed into vials to expand the culture
- Protein solutions containing different concentrations of 8F, M8 enzymes are ultrafiltered to concentrate proteins
- Expanded cultured bacteria containing the 28a-cl2-ADH gene were extracted for plasmids, extracted plasmids for concentration analysis, and run nucleic acid glue

Experimental equipment

- Plate colonies containing the 28a-cl2-ADH gene, pipette gun, tip of the pipette, . Ultra-clean workbench, alcohol lamp, marker
- 30000kd ultrafiltration tube, different concentrations of Mima's elution protein solution, Coomassie bright blue dyeing solution
- Escherichia coli containing 28a-c12-ADH, solution P1, P2, P3 rinse solution PW, adsorption column CP3, elution buffer EB

Experimental steps

① Pick bacteria

- On the ultra-clean workbench, 3 small bacteria liquid bottles were poured into the L stove sound nourishing base, and the medium was roughly half of the bottle body

- Use the tip to pick up individual colonies and transfer them into the vial
- Put the selected bacteria liquid bottle into a 37°C shaker and incubate for 8h

②ultrafiltration

- Select an ultrafiltration tube with a pore size of 30000kd and wash it with RO water
- Use RO water and buffe ultrafiltration for 1-2min (rinse + leak detection)
- The collected eluate was added to the inner tube of the ultrafiltration tube, and after leveling, it was centrifuged at 4000rpm 10°C for 5min
- The inner and outer tubes were tested separately using G250, and if the protein did not leak out of the inner tube, the outer tube liquid was poured and the corresponding eluent was added to the inner tube layer
- Repeat step 4 until the eluate is fully concentrated, and then add buffe ligan centrifugation to the ultrafiltration tube to remove the midset.
- Repeat twice with buffer centrifugation and continue centrifugation into the inner tube with a solution volume of 500-1000ul
- After ultrafiltration is completed, the concentrate in the inner tube is divided into 1.5ml EP tubes and marked accordingly, 200ul per tube
- Quick-freeze the aliquoted proteins with liquid nitrogen and store in a -80 °C freezer for use

Experiment Results

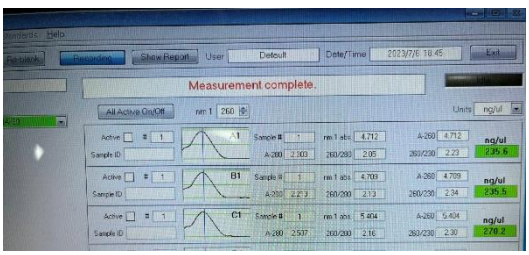


Figure1.Plasmid concentration test results

Transformation, measurement of 8F, M8 activity, pick bacteria

Friday, July 7, 2023

Overview

- 28a-c12-FDH conversion
- The activity of 8F and M8 was measured, of which the activity of 8F was ultra-high, the optimal PH was 6.76, and the activity of M8 was good, and the optimal PH was 5.58
- More than 2a single colonies were picked and the culture was expanded, because N23 almost did not grow on the plate, so it was not picked

Experimental equipment

- Competent E. coli, NZY medium, K medium
- Substrates 10 mM formic acid, 1 mM NAD+
- Ultra-clean workbench, alcohol bottle, alcohol lamp

Experimental steps

①Conversion of 28a-cl2-FDH

- Add competent cells 25u|, plasmid 1.5ul to a 1.5 ml EP tube in an ultra-clean bench
- Heat stir in a 42°C water bath for 45s, and then immediately ice bath for 2min
- Add NZY medium 150ul to the ultra-clean bench
- Incubate at constant temperature for 40min-60min in a shaker at 37 °C
- After incubation, 100ul of the bacterial solution was coated in LK resistant plates and cultured at 37°C

②8F, M8 activity was measured

- Configure buffers with pH 4.97, 5.58, 5.87, 5.97, 6.52, 6.76, 7.06, and 7.37

- Add 15ul1MNAD+, 15ul10mM formic acid, and 90ul buffers of different pH to the 1.5ml EP tube
- Transfer the above solution to the cuvette by pipette and zero (find the AUTO-ZERO button on the computer)
- After zeroing, add 30ul8F/30ulM8 protein solution to the cuvette and quickly press the 'measure' button on the computer to start the measurement
- Stop the measurement after 3 min of the reaction and observe the curve
- The range of Abs variation in each group within the 60s interval was recorded

Experiment results

(Table 1: Activity of 8F under different pH conditions)

8F is active under different pH conditions									
PH	**5.97**	**6.52**	**6.52**	**6.76**	**6.76**	**6.76**	**7.06**	**7.37**	**7.37**
Abs(60s)	**0.696**	**0.772**	**0.610**	**0.780**	**0.681**	**1.080**	**0.532**	**0.055**	**0.076**

(Table 2: Activity of M8 under different pH conditions)

M8 is active under different pH conditions									
PH	**4.97**	**5.58**	**5.87**	**5.87**	**5.97**	**5.97**	**6.52**	**6.76**	**7.06**
Abs(60s)	**0.480**	**0.515**	**0.300**	**0.478**	**0.357**	**0.487**	**0.219**	**0.123**	**0.170**

Pick bacteria, turn bottle & induce, electrophoresis

Saturday, July 8, 2023

Overview

- Plate singled colonies with the N23 protein scaffold gene were cultured in small bacterial vials
- E. coli containing the 28a-cl2-ADH gene cultured in small bacterial vials were transferred to large vials to expand the culture, and then induced to express the 28a-cl2-ADH protein
- Put 8F and M8 protein samples into 50, 60 and 70 °C water baths for 30 min

Experimental equipment

1. Pick bacteria

- Plate colonies containing N23 protein scaffold genes
- Pipette gun, tip
- Small bacterial bottles, LB medium
- Ampicillin
- Markers, workbenches, alcohol bottles, alcohol lamps

2: Turn bottle

- Bacterial vials culturing the 28a-cl2-ADH gene for E. coli
- Phosphate buffer (4x).
- kanamycin
- TB medium

3: Induce

- syringe
- IPTG

4: electrophoresis

- 8F, M8 enzyme, 1kbmaker, loading, PBS buffers for water baths at different temperatures
- Protein gel, electrophoresis instrument

Notes

- 1. When picking bacteria, the tip should not touch the mouth and wall of the bacterial bottle

- 2. Set the current when running glue

Experimental steps

1. Pick bacteria:

- 1. In the ultra-clean bench, add 50ul ampicillin to 100ml LB medium to make it LA medium
- 2. Pour one-third of LA medium into the small bacteria bottle
- 3. Use the tip to pick out a single colony and move it into the bacterial bottle
- 4. Put the selected small bacteria liquid bottle into a 37°C shaker and incubate for 8h

2. Transfer bottle:

- 1. Before receiving bacteria, place the required items (sterilization solution) in the ultra-clean workbench and irradiate ultraviolet for 10min
- Add 25ml of 4x phosphate buffer and 500ul of 2000x kanamycin to the two TB mediums, corresponding to one-half of the 28a-cl2-ADH gene solution, and label the bottles.
- The sterilized TB medium was incubated in a constant shaker at 37°C for 6-8h

3. Induction:

- Add 2000xIPTG 500 ul to the cultured bacterial solution and induce it at 18 °C for 16-18 h

4. electrophoresis:

- 1. Add 10ul of 8F and M8 enzymes to the 1.5ml EP tube, and put them into a water bath at 50, 60 and 70 °C for 30min
- 2. After the water bath, centrifuge for 1 minute at 12,000 rpm
- 3. Add 5ul supernatant, 35ulPBS buffer, 10ulloading, and metal bath to the 1.5ml EP tube for sample preparation
- 4. After sample preparation, the target protein is tested by SDS-PAGE. During the loading process, take 10ul for each sample and 2ul for protein marker.

Experimental results

- The cultured bacteria are growing well
- The analysis of running glue yielded: 8F protein single band, high enzyme activity, more M8 heteroprotein, average activity

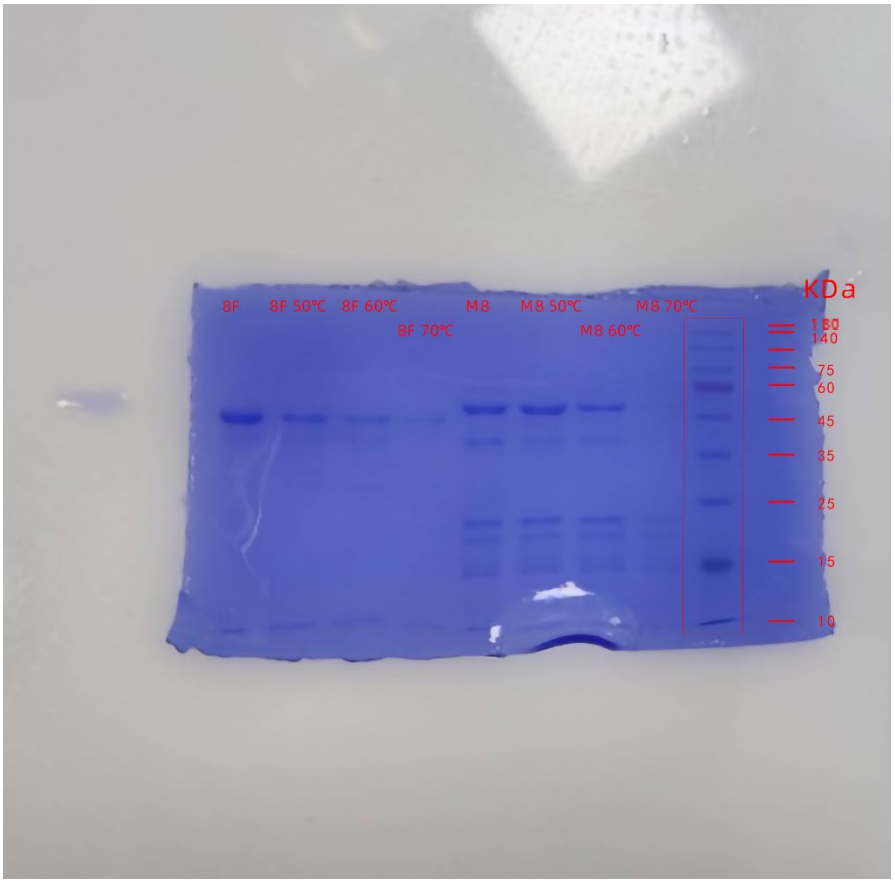


Figure 1: Results of gel electrophoresis

(3) Induction

- Inducer IPTG
- 1 ml syringe

Notes

(1) Transfer bottle

- Remember to pass the flame over the mouth of the bottle for each operation
- When injecting ammonanthicillin, the pipette body should not reach into the bottle mouth

(2) Harvest bacteria

- Pay attention to trimming

(3) Conversion

- The inducer IPTG is 500 u
- After injection, the medium is cultured in a shaker at 18 °C

Experimental steps

(1) Transfer bottle

- Before receiving bacteria, place the required items (sterilization solution) in the ultra-clean workbench and irradiate ultraviolet for 10min
- Add 4x phosphate buffer 25m|, ammoniapenicillin 500u|, and all bacteria to the TB medium, and label the bottle
- The sterilized TB medium was incubated in a constant temperature shaker at 37°C for about 9h

(2) Harvest bacteria

- Divide the bacterial liquid in the large bottle into the collection bottle, and the two pairs are flat
- After 6,000 rpm in a centrifuge for 10 min, pour the supernatant
- Resuspend the centrifuged bacteria with buffe and transfer to a 50 ml centrifuge tube with pair-equalization
- Centrifuge at 4000rmp at 10 °C for 10 min and then take out
- Pour the supernatant, weigh the dry weight and mark it, and store it in the refrigerator at

-20 °C

(3) Induction

- IPTG 500u| was added to the cultured bacterial solution and induced for 16-18 h at 18 °C

Sterilization (2a, A2), purification, Gel electrophoresis

Saturday, July 8, 2023

Overview

- E. coli containing 28a-ADH-cl2 and 28a-cl2-ADH are ruptured, releasing the enzyme protein of interest, and then the enzyme protein is collected
- The protein of interest was eluted by affinity chromatography and different concentrations of imidazole to purify the protein.
- Run the eluted protein solution

Experimental equipment

1. Sterilization:

- Low temperature autoclave sterilizer
- Pure water, phosphoric acid buffer
- 2 ml EP tubes
- A2, 2a bacterial solution
- Water bath

2. Purification:

- Nickel beads, different concentrations of imidazole
- PBSbuffer
- pmsf
- G250 Coomassie Bright Blue

3. Gel electrophoresis:

- Different concentrations of imidazole protein solution, 10-18kdmarker, loading
- Protein gel, electrophoresis instrument

Notes

1. Sterilization:

- The pressure of washing the machine and breaking the sterilization is different, remember to adjust

2. Purification

- Each elution should be sampled in time to react with G250
- Each bottle of eluate should be supplemented with the corresponding protease inhibitor PMSF and stored in ice

Experimental steps

1. Sterilization

- Bacteria are weighed on the balance and buffers are added to them for resuspendance
- Before sterilization, add the 1% protease inhibitor PMSF to the resuspended bacterial solution
- Wash the high-pressure cell disruptor twice with RO water and twice with PBSbuffer, keeping the pressure at 300bar-400bar during cleaning
- Add bacteria to the instrument, and the sterilization pressure is maintained at 1000bar-1200bar
- Repeat the sterilization 3-6 times so that the bacterial solution is no longer viscous
- After the sterilization is completed, aliquot with 2ml EP tubes, put them into a 60°C water bath for 10min (leave two tubes without water baths for sample preparation)
- After the water bath, put it into a centrifuge at 14000rpm 10 °C for 30min (one tube without water bath should be centrifuged, and the other tube should be centrifuged neither water bath nor centrifugation)
- After centrifugation, the supernatant and precipitate of the centrifugation without a water bath are retained, and the supernatant and precipitate of the centrifugation is also retained in a tube of water bath for sample preparation. The rest can be collected in the supernatant.

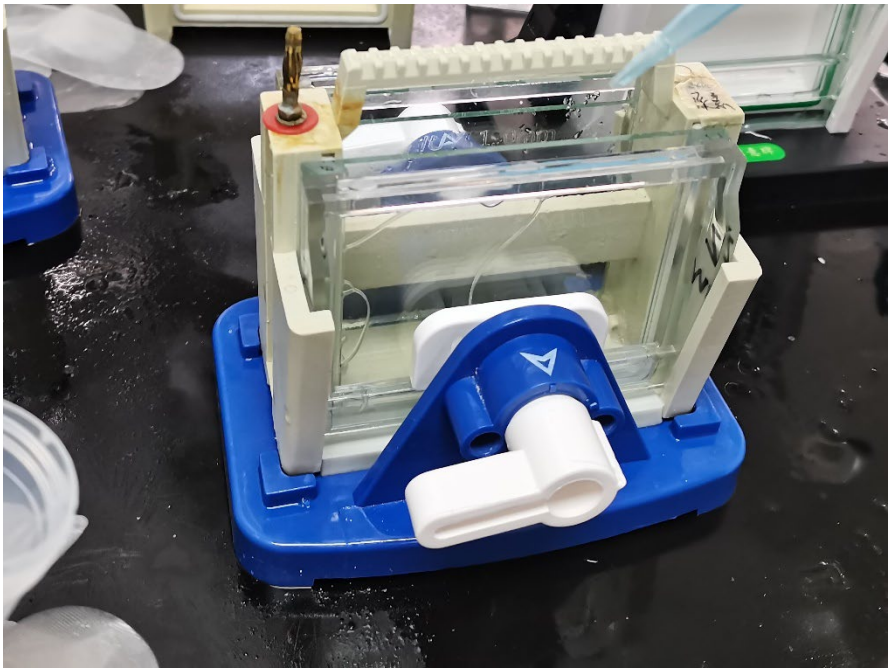
2. Purification

- An appropriate amount of nickel beads is first added to the purification column and then washed twice separately with RO water and PBSbuffer.
- After cleaning, an appropriate amount of 10mM imidazole was added to the purified beads with nickel beads and soaked for 5min to activate the nickel beads.
- After activating the nickel beads, immediately add the collected upper clearing night, and close the tube mouth with parafilm and mix in a silent mixer for 60 min (so that the protein of interest fully binds to the nickel beads).
- After mixing, take the flow penetration solution and place it in ice.
- Elution was performed with 10 mM imidazole, 30 mM imidazole, 50 mM imidazole, 80 mM, 100

mM imidazole, 150 mM imidazole, 300 mM imidazole, and 600 mM imidazole. Each concentration of imidazole is washed until the eluate encounters G250 Coomassie brilliant blue and does not change blue.

3. Gel electrophoresis

- Samples were prepared for precipitate and supernatant of collected boiled samples, precipitation and supernatant of uncooked samples, flow-through, whole, and various concentrations of imidazole eluate. Take 10ul loading and 40ul samples for mixing, and boil at 100°C for 10min.
- After sample preparation, the protein of interest is tested by SDS-PAGE. During the loading process, take 10ul for each sample and 2ul for protein marker.



Experimental results (supplemental)

Purification results:

- 2a Each concentration of imidazole is only eluted below, and no protein is eluted
- A2 elutes several times for each concentration of imidazole before no protein elution occurs

1. Analysis of gel electrophoresis results:

- 2a: There is no protein band, no target protein, it should be caused by picking bacteria error
- A2: 30-300 mM imidazole-eluted protein solution with protein band of interest

A2 protein ultrafiltration, simple activity measurement

Wednesday, July 12, 2023

Overview

- The running results showed that there were more A2 proteins, and the protein solution eluted by different concentrations was ultrafiltered to concentrate the target protein, and the obtained protein was simply detected for activity

Experimental equipment

- Ultrafiltration tubes, centrifuges
- Different concentrations of Mimajesty eluted protein solution, PBS buffer, G250 stain
- Pipette gun, tip of the pipette
- 10 mM methanol, 1 mMNAD+
- Wash the bottle

Notes

- The number of elution times of the last PBS solution is determined according to the amount of egg mortar solution
- One tube of concentrated protein liquid is about 1OOOul
- The concentration system is finally added with enzymes, and it is zeroed before the measurement

Experimental steps

① ultrafiltration

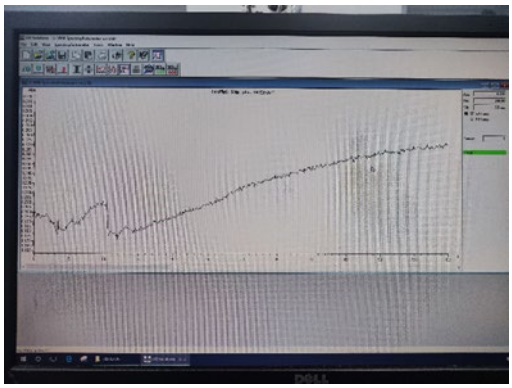
- Choose a 30,000-pore ultrafiltration tube and wash it with RO water
- Ultrafiltration with RO water and buffer for 1-2min (rinsing + leak detection)
- The collected eluate was added to the inner tube of the ultrafiltration tube, and centrifuged at 4000 rpm at 10 °C for 12 min in the 1000 rpm centrifuge after leveling
- The inner and outer tubes were tested separately using G250, and if the protein did not leak out of the inner tube, the outer tube liquid was poured and the corresponding eluent was added to the inner tube layer
- Repeat step 4
- After the eluate is fully concentrated, PBS is added to the ultrafiltration tube for centrifugation to remove Mima
- After 5 repeats, continue centrifugation into the inner tube with a solution volume of about 1 ml
- After ultrafiltration, the concentrate in the inner tube was aliquoted with 1.5ml EP tubes, and a mark was written on the tube every 200u|
- Quick-freeze the aliquoted egg mortars with liquid nitrogen and store in a -80 °C freezer for use

Ultrafiltration concentrates the finished A2 protein

(2) Test activity

- Open the software and set the measurement parameters and the address where the data will be saved
- First, add 15ul 1mM NAD+, 15ul 10mM methanol, and 90ul pbs to the 1.5ml EP tube, respectively
- Transfer the above solution to the cuvette by pipette and zero in
- After zeroing, add 30ul of protein solution to the cuvette and quickly press the 'measure' button on the computer to start the measurement
- After 2min-3min of the reaction, the stun measurement can be stopped and the curve can be observed

A2 system 1 measured activity



A2 system 2 measured activity

Experimental results

- A2 protein solution is divided into more than 10 tubes, each tube is 200ul
- The protein of the ultrafiltration inner tube was used to measure the activity, and 50ul protein was used in System-1 and 30ul protein was used in System-2, both of which were active, but 1 was higher than 2

Determination of hybrid system activity

Friday, July 14, 2023

Overview

- The four enzymes A2, F7, 8F and M8 were divided into two groups, and the activity at different pH was measured together

Experimental equipment

- A2, F7, 8F, M8 four proteases
- Substrate 10 methanol
- 1mMNAD+
- Buffers of different pH

Experimental steps

- The four enzymes A2, F7, 8F and M8 were divided into A (A2, F7, 8F), B (A2, F7, M8) two groups
- First, add 15ul1MNAD+, 15ul10mM methanol, and 90ulpH 4.94 buffers to the 1.5ml EP tube
- Transfer the above solution to the cuvette by pipette and zero (find the AUTO-ZERO button on the computer).
- After zeroing, add 30ulA2 protein solution to the cuvette. Quickly press the "measure" button on your computer to start measuring
- The reaction can stop the measurement after 2min-3min and observe the curve
- Swap 90ulbuffer with 60ulbuffer and 30ulF8 protein solution and repeat step 7 above Swap 60ulbuffer with 30ulbuffer and 30ul8F protein solution and repeat the above steps
- The buffer at pH 4.94 was replaced with a buffer with pH 5.58, 5.97 and 7.06, and the above steps were repeated, and the activity determination of group A was completed
- Group B activity determination, repeat the above operation

Results

- The optimum pH of the three enzyme mixtures is 5.58

When the activity of A2 protein solution was measured alone, Abs changed within 60 seconds

	A	B	C	D	E
1	time/pH	4.94	5.58	5.97	7.06
2	0s	0.635	1.046	0.411	0.276

A2 and F7 were mixed and the activity was measured, and Abs changed wit

	A	B	C	D	
1	time/pH	4.94	5.58	5.97	
2	0s	0.810	0.546	0.483	
3	60s	1.027	0.888	0.606	

A2, F7 and 8F were mixed and the activity was measured, and Abs changed within 60 s

	A	B	C	D	E
1	time/pH	4.94	5.58	5.97	7.06
2	0s	0.368	-0.436	-0.030	-0.875
3	60s	1.059	0.246	0.523	-0.836

The activity of A2, F7 and M8 was measured together, and Abs changed within

	A	B	C	D	E
1	time/pH	4.94	5.58	5.97	
2	0s	1.089	-0.273	0.465	
3	60s	1.755	0.440	1.145	

Concentrations of A2, F7, 8F and M8

were measured; the optimal mixed enzyme system was determined

Sunday, July 16, 2023

Overview

- The concentration of different enzymes is determined to determine the enzymes in each mixed system
- The activity of two sets of mixed enzyme reaction systems was measured

Experimental equipment

- UV spectrophotometer
- Four enzyme solutions, water, pH5.58 solution
- Pipette gun, tip of pipette, 1.5 ml EP tube
- Paper towels, wash bottles

Notes

- Clean the instrument before use
- The solution should be mixed well during the measurement

Experimental steps

① Enzyme concentration measurement

- Wash three times with water, pH5.58 solution, and the liquid only needs to cover the injection hole
- Add 2ul of enzyme samples sequentially, covering the lid
- Select the 340nm wavelength, click to measure, and record and photograph

after the circle image comes out

② Determine the optimal mixed enzyme system

- Wash the cuvette and add 150ulph5.58 solution to the control dish
- Add an appropriate amount of ph5.58 solution, 10mM methanol solution 15ul, 1mMNAD+15ul into the EP tube and mix well, and then add to another cuvette to zero
- It is divided into two groups of enzyme mixing system, each group of enzyme system has a different amount of enzyme, add it after mixing, and click the test
- After waiting for 90s, record the changed absorbance values and compare

Experimental results

① Different enzyme concentrations

Table1

Number of measurements/different enzyme concentrations (mg/ml).	A2	F7	8F	M8
1	10.37	9.23	1.70	3.89
2	9.94	9.46	1.32	3.76
mean	10.2	9.4	1.5	3.8

② Two sets of enzymes mixed system reactants and results

Table2						
Different systems (UL).	A2	F7	8F/M8	NAD+	-OH	PH5.8 solution
8F included	6.6	7.1	30	15	15	76.3
With M8	6.6	7.1	11.8	15	15	94.5

Table3		
Time (s)/ABS (340nm).	8F included	With M8
0	0.556	0.575
60	1.112	0.814
Variable value	0.554	0.239

(A2, F7, 8F) system is better

F7 ultrafiltration, F7, N23 conversion, A2 and mixed system optimal substrate concentration measurement

Tuesday, July 18, 2023

Overview

- The protein solution purified yesterday is ultrafiltered to increase the concentration of F7 protein
- Since there were very few F7 and N23 proteins obtained when purified yesterday, they were re-expressed and converted today
- The amount of substrate methanol was changed to explore the optimal substrate concentration for A2, A2, F7, and M8 mixed systems

Experimental equipment

① ultrafiltration

- Ultrafiltration tubes
- centrifuge
- 1xpbs buffer
- G250 Coomassie Bright Blue

② invert

- Competent E. coli
- NZY medium
- LA, LK medium

③ Optimal substrate concentration

- Purified proteins A2, F7, M8
- Different concentrations of methanol
- 1mMNAD+
- pH5.58buffer

Experimental steps

(1) F7 ultrafiltration

- The collected eluate was added to the inner tube of the ultrafiltration tube, and centrifuged in the centrifuge at 4000rpm 10°C for 5-10 min after leveling
- The inner and outer tubes were tested separately using G250, and if the protein did not leak out of the inner tube, the outer tube liquid was poured

and the corresponding eluent was added to the inner tube layer

- Repeat step 2
- After the eluate is fully concentrated, buffer is added to the ultrafiltration tube for centrifugation to remove imidazole
- After two repeats, continue centrifugation into the inner tube, where the solution volume is about 500-600ul
- After ultrafiltration, the concentrate in the inner tube was aliquoted with 1.5ml EP tubes, 100-200ul per tube
- Flash freeze the aliquoted proteins with liquid nitrogen and store in a -80 °C freezer

(2) F7, N23 conversion

- Add competent cells 25 ul and plasmid 1.5 ul with the 28a-FDH-Cl7 gene to a 1.5 ml EP tube in an ultra-clean bench and competent cells 25 ul and plasmid 1.5 ul with the N23 gene to the other EP tube
- Heat excitation: Water bath in a 42°C water bath for 45s, and then immediately ice bath for 2min
- Add 150 ul of NZY medium to each in the ultra-clean bench
- Incubate at constant temperature for 40min-60min in a shaker at 37 °C
- After incubation, 100ul of bacteria containing 28a-FDH-Cl7 gene was coated in LK resistant plates, and bacteria containing N23 genes were coated in LA resistant plates and reared overnight at 37°C

③ Optimal substrate concentration

1. A2 optimal substrate concentration:

- Prepare 10, 20, 30, 40, 50, 60, 70mM methanol solutions
- Add 15ul1MNAD+, 15ul10mM methanol, 103.4ulpH5.58buffer to 1.5ml EP tubes, respectively
- Transfer the above solution to the cuvette by pipette and zero (find the AUTO-ZERO button on the computer)
- After zeroing, add 16.8ul A2 protein solution to the cuvette. Quickly press the "measure" button on your computer to start measuring
- The reaction can stop the measurement after 2min-3min and observe the curve
- Change the methanol concentration to 20, 30, 40, 50, 60, 70mM, and repeat the above operation

2. The optimal substrate concentration of the mixing system:

- Add 15ul1MNAD+, 15ul10mM methanol, and pH 5.58buffer to a 1.5ml EP tube
- Transfer the above solution to the cuvette by pipette and zero (find the AUTO-ZERO button on the computer)
- After zeroing, A2, F7, M8 protein solution with a ratio of 1:1:1 is added to the cuvette. Quickly press the "measure" button on your computer to start measuring
- Change the methanol concentration to 20, 30, 60mM and repeat the above

operation

- When the methanol concentration in the system is 2mM, change the ratio of the three enzymes to 1:2:1 and repeat the above operation

Experimental results

① The optimal substrate amount for A2 is 6 mM

Table1								
Time (s)/concentration (methanol final concentration mM)	1	2	3	4	5	6	7	10
0S	0.391	0.472	0.449	0.468	0.412	0.349	0.417	0.335
ABS maximum value and time	0.623 (24.5S)	0.694 (9.1S)	0.782 (9.5S)	0.706 (32.0S)	0.806 （35.6S ）	0.831 (11.8S)	0.727 (22.7S)	0.515 (15.0S)
60S	0.543	0.420	0.483	0.598	0.696	0.609	0.619	0.400

② The hybrid system (three enzymes ratio 1:1:1) has an optimal substrate concentration of 2 mM compared to the addition of A2 protein alone

Table2				
Time (S)/concentration (methanol final concentration mM)	1	2	3	6
0S	0.558	0.613	0.385	0.328
60S	1.112	1.227	0.937	0.939

③ The mixed system (ratio of three enzymes 1:2:1), the methanol concentration in the system is 2 mM

Table3	
Time (S)/concentration (methanol final concentration mM).	2
0S	0.385
60S	0.743

F7, N23 to bottle, induce

Thursday, July 20, 2023

Overview

- Transfer F7,N23 to TB medium and expand the culture for 8-10 h, after which the inducer IPTG is added to induce its expression of the protein of interest

Experimental equipment

- 4xbuffe buffer, ammonia-dried penicillin, kanamycin
- TB medium
- Pipette gun, tip of the pipette
- IPTG

Notes

- Aseptic operation should be performed when transferring bottles
- After IPTG induction, the medium is transferred to 18° (shaker for culture

Experimental steps

- Before receiving bacteria, the required items (sterilization solution) were placed in the Qianchao clean workbench, and ultraviolet irradiation was 10m1n
- Add 4x phosphate buffer 25m|, ammoniapenicillin 500ul or antibiotics 500ul to the TB medium to make the corresponding resistance medium, half of the F7 bacterial liquid bottle, all the bacterial liquid of the N23

bacterial liquid bottle, and the bottle body is marked

- The sterilized medium was cultured in a constant shaker at 37°C for 6-Bh
- Add 500 ul of IPTG to each medium and transfer to an 18 °C shaker to induce for 16-18 h

to bottle

18℃ induce

Harvesting (F7, N23)

Friday, July 21, 2023

Overview

- Collect E. coli containing the 28a-FALDH-CL7 gene and the N23 gene after expanded culture

Experimental equipment

- Harvesting bottles, centrifuge tubes

- centrifuge
- PBSbuffer

Notes

- The harvesting bottle and centrifuge tube must be well matched

Experimental steps

- Divide the bacterial liquid in the large bottle into the collection bottle, and the two pairs are flat
- After 6,000 rpm in a centrifuge for 10 min, pour the supernatant
- Resuspend the centrifuged bacteria with PBSbuffer and transfer to 50 ml centrifuge tubes with pair-leveling
- Centrifuge in a centrifuge at 3800rmp 10°C for 20 min and then take out
- Pour the supernatant, weigh the bacteria, record it, and store at -20 °C in the refrigerator

Ultrafiltration and concentration measurement of F7、8F、N23

Sunday, July 23, 2023

Overview

- F7, 8F, N23 protein ultrafiltration concentration, determination of concentration, aliquot into -80 °C refrigerator for preservation
- Measure the concentration of different proteins

Experimental equipment

- 30000kd ultrafiltration tube
- Different concentrations of protein solution eluted by Mimajesty

- Pipette gun, tip of the pipette
- PBS buffer, G250 stain
- 1.5 ml EP tube

Experimental steps

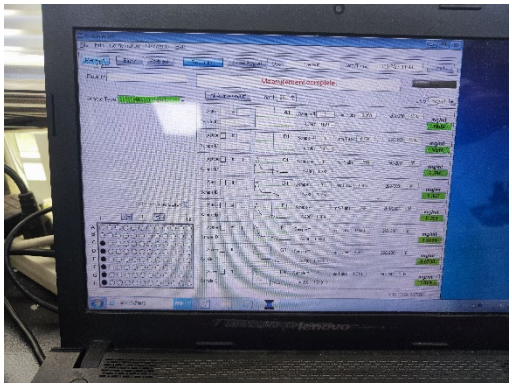
①ultrafiltration

- Choose a 30,000-pore ultrafiltration tube and wash it with RO water
- Use RO water and buffe ultrafiltration for 1-2min (rinse + leak detection)
- The collected eluate was added to the inner tube of the ultrafiltration tube, and centrifuged at 4000rpm 10°C for 10min after leveling
- The inner and outer tubes were tested separately using G250, and if the protein did not leak out of the inner tube, the outer tube liquid was poured and the corresponding eluent was added to the inner tube layer
- Repeat step 4
- After the eluate is fully concentrated, PBS is added to the ultrafiltration tube for centrifugation to remove Mima
- After 5 repeats, continue centrifugation into the inner tube with a solution volume of about 1 ml
- After ultrafiltration, the concentrate in the inner tube was aliquoted with 1.5ml EP tubes, and a mark was written on the tube every 200u|
- Quick-freeze the aliquoted proteins with liquid nitrogen and store in a -80 °C reezer for use

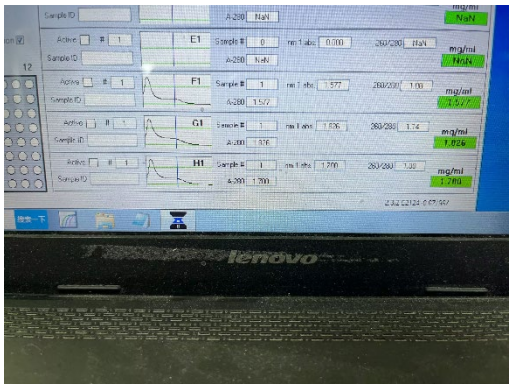
The protein solution to be ultrafiltered

②Measure concentration

- Wash three times with water and once with PBS solution, and the liquid only needs to cover the injection hole
- Add 2ul of enzyme samples sequentially, covering the lid
-
- Select the 340nm wavelength, click to measure, record and take pictures when the image is out



F7,N23 Concentration results



8F Concentration results

Concentration test results（mg/ml）

Protein	F7	8F	N23
Concentration	1.36	1.7	0.92

Mixed system activity assay (different enzyme ratios), transformation(A2, 2A)

7.24 Monday, 2023

Overview

- Change the ratio of A2, F7, 8F to perform activity determination
- Plasmids containing the 28a-ADH-CL2 gene and the 28a-CL2-ADH gene were transferred to competent E. coli, respectively

Experimental equipment

1. Activity determination

- Purified proteins A2, F7, 8F
- Different concentrations of methanol
- 1mMNAD+
- Different pH buffers

2. Conversions

- Plasmids containing the 28a-ADH-CL2 gene and the 28a-CL2-ADH gene
- Competent E. coli
- NZY medium
- Kanamycin-resistant plates

Experimental steps

(1) Activity determination

- Determine the amount of the three enzymes as 30ul and calculate the volume of each enzyme in the different proportions required
- First, add 15ul 1M NAD+, 15ul methanol, and an appropriate amount of different pHbuffers to the 1.5ml EP tube
- Transfer the above solution to the cuvette by pipette and zero (find the AUTO-ZERO button on the computer)
- The three enzymes are first mixed in a specific volume in a 1.5 ml EP tube, then transferred to a cuvette, and quickly press the "measure" button on the computer to start the measurement
- The reaction takes 2min-3min to stop the measurement and observe the curve

(2) Conversion

- Add competent cells 25ul, plasmid 1.5 ul (plasmids can be added to the table) to a 1.5 ml EP tube in an ultra-clean bench and ice bath for 30 min
- Heat Stirring: Bath in a 42°C water bath for 45s, then immediately ice bath for 2min
- Add NZY medium 150 ul to the ultra-clean bench
- Incubate at a constant temperature in a 37°C shaker for 40 min-60 min
- After incubation, 150ul of bacterial solution was applied to kanamycin-resistant plates and reared overnight at 37°C

Experimental results

① Negative control, buferpH 5.6(5.58) (15ul methanol, 15ulNAD+, specific volume of enzyme, buffer, 150ul system)

Time/Conditions	Methanol free (enzyme **1:1:1**).	only **NAD+** (enzyme **1:1:1**)	Only no enzymes	No methanol, **NAD+** (enzyme **1:1：1**)	No methanol, **NAD+** (enzyme **1:1：2**)	Free from methanol, **NAD+** (enzymes **1:2:2**)	Methanol-free, **NAD+** (enzyme **1:1:1 system mixed).**
0s	0.574	0.469	0	0.477	0.437	0.287	**1.449**
60s	1.341	1.165	0	1.038	1.585	1.756	**1.666**
Difference	**0.767**	**0.696**	**0**	**0.561**	**1.148**	**1.469**	**0.217**

② 2 mM methanol with different enzyme ratios (15ul methanol, 15ulNAD+, specific volume of enzyme, buffer, 150ul system)

Time/enzyme ratio	**1:1:1**	**1：1:2**	**1：1:3**	**1:2:2**	**1:2:3**
0s	0.529	0.015	0.757	0.376	**0.568**
60s	**0.938**	**0.954**	**1.741**	**1.539**	**2.161**

Difference	**0.409**	**0.939**	**0.984**	**1.163**	**1.593**
Subtract the	-	-	no	-	n

value of the negative control	**0.152**	**0.209**	t	**0.306**	ot

③ 2 mM methanol, pH different buffers (15ul methanol, 15ulNAD+, enzyme 1:1:1, buffer, 150ul system)

Time/pH	**5.6**	**6.5**	**7.0**	**7.0 (no methanol, NAD+, negative control).**	**7.5**
0s	0.529	0.540	0.230	0.214	**0.162**
60s	0.938	0.928	0.456	0.327	**0.189**
Difference	**0.409**	**0.388**	**0.226**	**0.113**	**0.027**

④ Different concentrations of methanol (15ul methanol, 15ulNAD+, enzyme 1:1:1, buffer, 150ul system)

Time/methanol concentration (mM)	**1**	**2**	**3**	**6**
0s	0.537	0.529	0.316	**0.297**
60s	0.749	0.938	0.689	**0.427**
Difference	0.212	0.409	0.373	**0.130**
Subtract the negative control value	**-0.349**	**-0.152**	**-0.188**	**-0.431**

7.25 Single enzyme activity assay (pH different)

Monday, July 25, 2023

Overview

- Change the pH of the A2, F7, 8F three enzyme systems, and do negative control experiments without substrate and NAD+ at the same time to perform activity determination

Experimental equipment

- Purified proteins A2, F7, 8F
- 1mM methanol, 1mM formaldehyde, 1mM formic acid
- 1mMNAD+
- Different pH buffers

Experimental steps

- Determine the concentration of the three enzymes and calculate the volume of each enzyme at 30ug
- First, add 15ul 1M NAD+, 15ul methanol, and an appropriate amount of different pHbuffers to the 1.5ml EP tube
- Transfer the above solution to the cuvette by pipette and zero (find the AUTO-ZERO button on the computer)
- Add A2 to a 1.5ml EP tube in a specific volume to mix, then transfer to a cuvette, quickly press the "measure" button on the computer to start the measurement, react 2min-3min to stop the measurement, observe the curve

- Change the pH and enzyme repeat the experiment
- In negative control experiments, only PBS and enzymes were added to measure the activity at each pH

Experimental results

① A2 (negative control is formaldehyde-free, NAD+, 150ul system)

Time/PH	5.59	negative	5.97	negative	6.52	negative	7.06	negative	7.57	negative
0s	0.180	0.048	0.036	0.123	0.135	0.188	0.043	0.069	-0.017	0.031
60s	0.514	0.130	0.163	0.193	0.138	0.067	0.018	0.014	-0.017	0.031
Difference	0.334	0.082	0.127	0.070	0.003	-0.121	-0.025	-0.055	0	0
Actually poor	0.252		0.057		0.124		0.030		0	

② F7, (negative control is formic acid-free, NAD+, 150ul system).

Time/PH	5.59	negative	5.97	negative	6.52	negative
0S	-0.042	0.098	0.099	0.066	0.110	0.046
60S	0.137	0.240	0.323	0.178	0.270	0.051
Difference	0.179	0.142	0.224	0.112	0.160	0.005
Actually poor	0.037		0.112		0.155	

③ 8F, (negative control is formic acid-free, NAD+, 150ul system)

Time/PH	5.59	negative	5.97	negative	6.52	negative	7.06	negative	7.57	negative
0s	0.419	0.532	0.429	0.403	0.285	0.144	0.027	0.012	0.013	0.007
60s	0.888	0.899	0.821	0.802	0.678	0.426	0.235	0.100	0.016	0.007
Difference	0.469	0.367	0.392	0.399	0.393	0.282	0.208	0.088	0.003	0
Actually poor	0.102		-0.007		0.193		0.120		0.003	

7.27 A2, 2A, M8 pick bacteria, transfer bottle, induce: A2, F7, M8 mixed with N23, through molecular sieve, run glue

Thursday, 7.27 2023

Overview

- A2, 2A, M8 were picked and bottled to expand the culture and induce them to express the corresponding protein
- The three groups of dehydrogenase A2, F7, 8F obtained were combined with protein scaffold N23, and the bound enzyme complex was screened out by molecular sieve, and the results were determined by running rubber

Experimental equipment

- LK medium, bacterial bottles, markers, pipette guns, tips
- TB medium, kanamycin, ampicillin, 4×phosphate buffer buffer, IPTG

- PBS buffer, 20% ethanol, UP water, AKTA protein purifier
- Loading，maker

Experimental steps

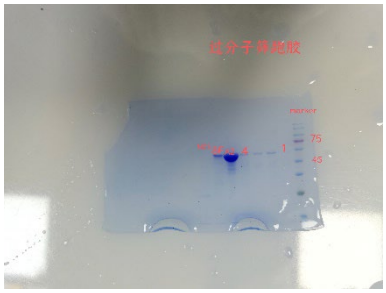
1. A2, 2A, M8 provoke, bottle transfer, induce

- Pour the corresponding resistance medium into the vial of small bacteria on the bench, and the medium does not exceed 1/3 of the vial
- Carefully pick 3-4 single colonies with the tip of the gun, move them into the corresponding small bacteria bottle, shake well and mark them accordingly
- Place in a 37 °C shaker and incubate for 6-8 h
- Before receiving bacteria, place the required items (sterilization solution) in the ultra-clean workbench and irradiate ultraviolet for 10min
- Add 25ml of 4x phosphate buffer, 500ul of kanamycin, and 1/2 of the corresponding bacterial liquid bottle to the TB medium, and mark the bottle
- The sterilized medium was incubated in a constant temperature shaker at 37°C for about 8h
- Add IPTG 500ul to the cultured bacterial solution and induce it at 18 °C for 16-18 h

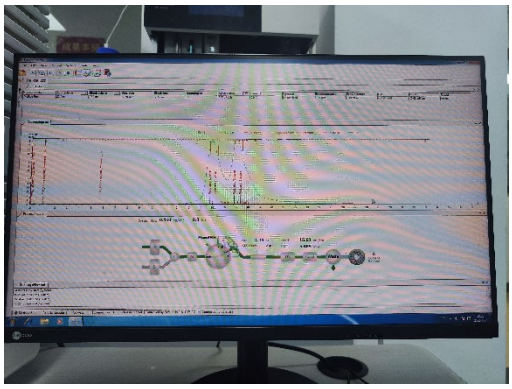
Pick bacteria

2. A2, F7, 8F mixed with N23, through molecular sieve, running glue

- Mix 100ul A2, 200ul F7, 8F, N23, incubate at 25°C for 10min, and put on ice for later use
- Open the AKTA protein purifier and set the corresponding parameters: select A pump, pressure 2Mpa, flow rate 0.4ml/min, time 100min
- Wash with UP water and PBS for 100min, respectively
- Load the sample with a 1 ml syringe and connect with a 1.5 ml EP tube
- According to the sequential numbering, and prepare the sample running glue, 10ulloading + 20ulpbs + 20ul sample
- The prepared sample metal bath was 100 °C for 10min, and the sample ran glue



SDS-PAGE result



Molecular sieve out peak diagram

7.28 Harvesting (A2, 2A, M8)

Friday, 7.28 2023

Overview

- Collect E. coli containing A2, 2A, and M8 protein genes after expanded culture

Experimental equipment

- Harvesting bottles, centrifuge tubes
- centrifuge
- PBSbuffer

Experimental steps

- Divide the bacterial liquid in the large bottle into the collection bottle, and the two pairs are flat
- After 6,000 rpm in a centrifuge for 10 min, pour the supernatant
- Resuspend the centrifuged bacteria with PBSbuffer and transfer to 50 ml centrifuge tubes with pair-equalization
- Centrifuge in a centrifuge at 4000rmp 10 °C for 15 min and then take out
- Pour the supernatant and store at -20 °C in the refrigerator

7.29 A2, M8 sterilization, purification, running glue

Saturday, 7.29 2023

Overview

- The bacteria containing A2 and M8-related proteins were broken, eluted and collected with different concentrations of imidazole, and then the collected proteins were analyzed for glue

Experimental equipment

- PMSF protease inhibitors, imidazole solutions in different concentrations
- Buffer buffer, A2, M8 bacterial solution
- 2 ml EP tube, 10 ml EP tube, G250 dye
- Maker，loading
- Pipette gun, tip of the pipette

Experimental steps

(1) Sterilization

- Bacteria are weighed on the balance and 1 g of bacteria are added to 10 ml buffer for resuspendance
- Before sterilization, add the 1% protease inhibitor PMSF to the resuspended bacterial solution, the volume is ten times the grammage
- Wash the high-pressure cell disruptor twice with RO water and twice with xbuffer, maintaining the pressure at 300bar-400bar during cleaning
- Add bacteria to the instrument, and the sterilization pressure is maintained

at 900bar-1100bar

- Repeat the sterilization 3-4 times so that the bacterial solution is no longer viscous
- After the sterilization is completed, aliquot with 2ml EP tubes and centrifuge in a centrifuge at 12000 rpm at 10 °C for 30 min (leave one tube without centrifugation for sample preparation).
- After centrifugation, one tube of pellet and one tube of supernatant (sample preparation) are retained. The rest can be collected in the supernatant

破菌

(2) Purification

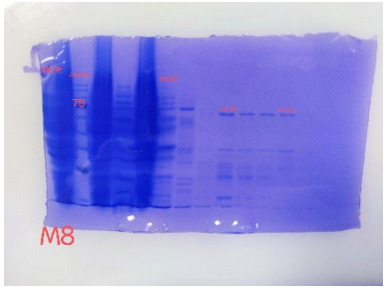
- Wash the purification column with tap water and RO water
- The purification column was soaked with NaOH at a concentration of 0.1 M for 15 min
- Wash away NaOH from the purification column and rinse with RO water
- First add an appropriate amount of nickel beads to the purification column and wash twice separately with RO water and buffer.
- After cleaning, an appropriate amount of 10mM imidazole was added to

the purified beads with nickel beads and soaked for 5min to activate the nickel beads.

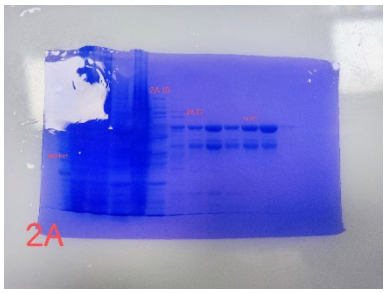
- After activating the nickel beads, immediately add the collected upper clear night, and close the tube mouth with parafilm and mix in a silent mixer for 90 min (so that the protein of interest fully binds to the nickel beads).
- After mixing, take the flow penetration solution and place it in ice.
- Elution was performed with 10 mM imidazole, 30 mM imidazole, 50 mM imidazole, 80 mM imidazole, 100 mM imidazole, 150 mM imidazole, 300 mM imidazole, and 600 mM imidazole. Each concentration of imidazole is washed until the eluate encounters G250 Coomassie brilliant blue and does not change blue

(3) Run glue

- Samples were prepared for the collected precipitate, supernatant, flow-through, whole, and various concentrations of imidazole eluate. Take 40ul loading and 10ul samples for mixing, and boil at 100°C for 10min.
- After sample preparation, the protein of interest is tested by SDS-PAGE. During the loading process, take 10ul for each sample and 2ul for protein marker.



M8 SDS-PAGE result



2A SDS-PAGE result

7.30 Ultrafiltration (2A)

Sunday, 7.30 2023

Overview

The purified protein liquid is centrifuged and concentrated

Experimental equipment

- Ultrafiltration tubes
- centrifuge
- PBSbuffer
- 2A protein solution
- G250

Experimental steps

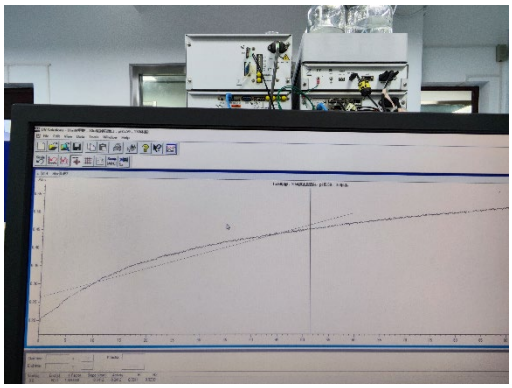
- The collected various types of eluate were added to the inner tube of the ultrafiltration tube (leaving a part of the eluate for activity determination), and centrifuged in a centrifuge at 4000rpm 10 °C for 15min
- The inner and outer tubes were tested separately using G250, and if the protein did not leak out of the inner tube, the outer tube liquid was poured and the corresponding eluent was added to the inner tube layer
- Repeat step 2

- After the eluate is fully concentrated, buffer is added to the ultrafiltration tube for centrifugation to remove imidazole.
- After two replicates, continue centrifugation into the inner tube with a solution volume of less than 1 mL
- After ultrafiltration, the concentrate in the inner tube was mixed and aliquoted with 1.5ml EP tubes, 100-200ul per tube
- Leave one tube to measure the activity, and the other liquid nitrogen is used to quick-freeze the aliquoted protein and store in the -80 °C freezer for use
- The concentration of the remaining ultrafiltered protein solution was measured and the activity was determined

Experimental results

- The concentration of the resulting ultrafiltered 2A protein solution was 10.15mg/ml and was active

Measure the concentration of 2A protein



2A simple activity measurement