**Construction of endoglucanase Cel5A display vector**

Mar 31 Receive plasmid cel5A-pMD 18-T

Apr 4 Transform cel5A-pMD 18-T into DH5α competent cells. Incubate overnight at 37℃.

Apr 5 (1) Pick monoclonal transformants for streak cultivation on another LB culture plate, incubate at 37℃ for 10h.

(2) Pick 8 monoclonal transformants from the plate and inoculate in 5ml liquid LB medium. Incubate over night at 37℃ on a shaking incubator.

Apr 6 (1) Take 800ul of each transformant for glycerol conservation at -80℃.

(2) Extract plasmid from cel5A-pMD 18-T transformants.

(3) Send plasmids from two transformants for sequencing.

Apr 8 Seqence of cel5A-pMD 18-T correct.

May 20 Incubate 100ul cel5A-pMD 18-T strain with the correct sequence in 5ml liquid LB medium.

May 21 (1) Take 800ul cel5A-pMD 18-T for glycerol conservation.

(2) Incubate *Pichia pastoris* surface display vector 9k-ES-4 in liquid LB medium.

May 22 Glycerol conservation for 9k-ES-4. Plasmid extraction.

Jun 6 Receive primers cel5A-EHAM-F/S, dilute with $ddH_2O$ to 10uM.

Jun 7 Temperature gradient PCR with cel5A-pMD 18-T as template, cel5A-EHAM-F/S as primers to explore the optimum annealing temperature. Results at all annealing temperatures show fragmental bands.

Jun 8 (1) Raise annealing temperatures and repeat the PCR of yesterday. Results show that the optimal temperature for annealing is 62℃.

(2) PCR with cel5A-pMD 18-T as template and cel5A-EHAM-F/S as primers, extract PCR products on gel, and name as cel5A-EHAM.

(3) Digest cel5A-EHAM and 9k-ES-4 with EcoRI, MluI

Jun 9 Gel extraction of digestion product of 9k-ES-4.

Jun 12 Ligate cel5A-EHAM and 9k-ES-4 vector digested on June 8.

Jun 13 Transform the ligated product from yesterday into DH5α competent cells.

Jun 14 Transformation fails.

Jun 19 Ligate cel5A-EHAM with 9k-ES-4 vector and transform into DH5α competent cells again.

Jun 20 (1) Transformation fails.

(2) Water bath for cel5A-EHAM at 80℃ for 5min then ligate with 9k-ES-4 vector. Transform into DH5α competent cells. Problem occurs.

Jun 21 Repeat step (2) of yesterday.

Jun 22 Colony PCR with cel5A-EHAM-F/S as primers. Inbubate transformants with the correct colony PCR results.

Jun 23 (1) Glycerol conservation for cel5A-9k-ES-4, extract plasmid.

(2) Digest with EcoRI, MluI. Bands confirmed.

(3) Transformants with both correct colony PCR and enzyme digestion are sent for sequencing.

Jun 25 Sequence correct.

**Construction of β-glucosidase bglX display vector**

May 21 Incubate *Pichia pastoris* strain with surface display vector ZXFα in liquid LB medium.

May 22 Glycerol conservation of ZXFα strains, extract plasmid.

Jun 9 Transform plasmid bglX-pSB1C3 into DH5α competent cells. Incubate overnight at 37℃.

Jun 10 Pick colonies of bglX-pSB1C3 for shaking incubation.

Jun 11 Extract plasmids from bglX-pSB1C3 transformants. Digest with EcoRI, PstI.

Jun 12 bglX-pSB1C3 digestion fails.

Jun 18 (1) Temperature gradient PCR with bglX-pSB1C3 plasmid as template, VF2/VR as primers. Result is confirmed, having specific bands.
(2) Digest bglX-pSB1C3 again with EcoRI, PstI.
(3) Send the plasmid of a bglX-pSB1C3 transformant for sequencing.

Jun 19 Digestion of bglX-pSB1C3 fails.

Jun 20 Sequencing of bglX-pSB1C3 correct.

Jul 19 Receive bglX-NX-F/S primers, dilute with $ddH_2O$ to 10uM.

Jul 20 (1) Temperature gradient PCR with bglX-pSB1C3 as template and bglX-NX-F/S as primers. Result shows that optimum annealing temperature should be 61℃.
(2) PCR at 61℃ with bglX-pSB1C3 as template and bglX-NX-F/S as primers.
(3) Gel extract PCR product.
(4) Digest gel extraction product and ZXFα using NheI and XhoI.

Jul 21 Digestion of ZXFα fails. Re-incubate ZXFα strains.

Jul 22 (1) Extract plasmid from ZXFα strains.
(2) Digest ZXFα using NheI and XhoI. Digestion succeeds, gel extract vector.
(3) Water bath for digestion product of bglX from July 20 at 80℃ for 5min to inactivate restriction endonuclease.
(4) Ligate bglX with ZXFα.

Jul 23 Transfer bglX-ZXFα into DH5α competent cells.

Jul 24 Pick monoclonal transformants from the culture and transfer onto another plate.

Jul 25 (1) Pick colonies from the plate above for colony PCR. Results all negative. Pick transformants for incubation.
(2) PCR with bglX-pSB1C3 as template and bglX-NX-F/S as primers with Tm of 61℃.
(3) Gel extract PCR product.

Jul 26 (1) Conserve bglX-ZXFα strains and extract plasmid. Digest using NheI and XhoI, fail. Transformation fails.
(2) Ligate bglX with pMD18-T vector. Transform ligation product into DH5α competent cells.

Jul 27 (1) Pick monoclones from the transformation plate for streak cultivation on another plate.
(2) Pick colonies from the plate for colony PCR.

Jul 28 Agarose gel electrophoresis result of colony PCR positive. Pick 3 transformants for incubation.

Jul 29 Conserve bglX-pMD18-T strains, extract plasmid. Digest with NheI and XhoI, result correct.

Jul 30 Digest bglX-pMD18-T plasmid and ZXFα vector, gel extract the digested bglX and ZXFα. Ligate both and transform into DH5α competent cells.

| | |
|---|---|
| Jul 31 | Pick monoclones for colony PCR. |
| Aug 1 | Conserve bglX-ZXFα strain, extract plasmid. Ligate with NheI and XhoI, with correct results. |
| Aug 2 | Send plasmids with both correct bglX-ZXFα colony PCR and digestion results for sequencing. |
| Aug 4 | Sequencing correct. |

**Construction of xylanase xyn display vector**

Apr 25 Receive plasmid xyn-pET-28a.

May 20 Transform plasmid xyn-pET-28a.

May 21 (1) Pick monoclonal transformants for streak cultivation on another LB culture plate, incubate 10h at 37℃.

(2) Pick 4 monoclonal transformants from the plate and inoculate in 5ml liquid LB medium, incubate overnight at 37℃.

(3) Incubate *Pichia pastoris* strains with surface display vector ZXFα in liquid LB medium.

May 22 (1) Glycerol conservation of 4 monoclonal transformants of xyn-pET-28a, extract plasmid.

(2) Glycerol conservation of ZXFα, extract plasmid.

Jun 4 Send 2 transformant plasmids of xyn-pET-28a for sequencing.

Jun 6 Sequencing of xyn-pET-28a correct. Receive primers xyn-NX-F/S, dilute with $ddH_2O$ to 10uM.

Jun 7 Temperature gradient PCR with xyn-pET-28a as template and xyn-NX-F/S as primers to explore the optimal annealing temperature. Results of all annealing temperatures show fragmental bands.

Jun 8 (1) Raise annealing temperature, repeat PCR of yesterday. Results show that the optimal temperature for annealing is 61℃.

(2) PCR with xyn-pET-28a as template and xyn-NX-F/S as primers. Fail.

Jun 9 Repeat PCR of yesterday twice. Both fail.

Jun 11 Repeat PCR of June 8, fail.

Through reading literature and sequence alignment, we find that the xyn gene we obtained is a mature fragment devoid of signal peptide. Thus the design of primers xyn-NX-F/S is erroneous.

Jul 19 Receive newly designed xyn(M)-NX-F/S primers for amplifying mature xyn sequence. Dilute with $ddH_2O$ to 10uM.

Jul 20 (1) Temperature gradient PCR using xyn-pET-28a as template and xyn(M)-NX-F/S as primers.

(2) Gel extract PCR product.

(3) Digest PCR product and ZXFα vector using NheI and XhoI.

Jul 21 Digestion of ZXFα fails. Re-incubate ZXFα strain.

Jul 22 (1) Extract plasmid from ZXFα strain.

(2) Digest ZXFα using NheI and XhoI. Digestion succeeds, gel extract vector.

(3) Water bath xyn digestion product from July 20 at 80℃ for 5min to inactivate restriction endonuclease.

(4) Ligate xyn with ZXFα.

Jul 23 Transform xyn-ZXFα into DH5α competent cells.

Jul 24 Pick monoclones from the transformation plate for streak cultivation on another plate.

Jul 25 (1) Pick monoclonal transformants for colony PCR. All results negative. Pick transformants for incubation.

(2) PCR with xyn-pET-28a as template and xyn（M）-NX-F/S as primers.

(3) Gel extract PCR product.

Jul 26 (1) Conserve xyn-ZXFα transformants, extract plasmid. Digest plasmid using NheI and XhoI, digestion fails, indicating that transformation fails.

(2) Ligate xyn with pMD18-T vector. Transform into DH5α competent cells.

Jul 27 (1) Pick monoclones from transformation plate for streak cultivation on another plate.

(2) Pick monoclonal transformants from the plate for colony PCR.

Jul 28 Agarose gel electrophoresis result of colony PCR shows positive. Pick 3 transformants for incubation.

Jul 29 Conserve xyn-pMD18-T transformant strain, extract plasmid. Digest using NheI and XhoI, results correct.

Jul 30 Digest xyn-pMD18-T and ZXFα using NheI and XhoI. Gel extract digested xyn gene frangment and ZXFα vector fragment, ligate both and transform into DH5α competent cells.

Jul 31 Pick monoclones for colony PCR.

Aug 1 Conserve xyn-ZXFα strain, extract plasmid. Digest using NheI and XhoI, correct.

Aug 2 Plasmids with both correct xyn-ZXFα colony PCR result and digestion are sent for sequencing.

Aug 4 Sequencing correct.

**Construction of β-xylosidase Ruxyn display vector plasmid**

May 21 Incubate *Pichia pastoris* strain with surface display vector pPIC9k-ES-4 in liquid LB medium.

May 22 Glycerol conservation of 9k-ES-4, extract plasmid.

Jun 12 Receive Ruxyn-pET-21a plasmid.

Jun 13 Transform Ruxyn-pET-21a into DH5α competent cells. Incubate overnight at 37℃.

Jun 14 (1) Pick monoclonal transformants for streak cultivation on another LB plate. Incubate 10h at 37℃.

(2) Pick 3 monoclonal transformants from the plate and inoculate in 5ml liquid LB medium. Incubate overnight at 37℃.

Jun 15 Glycerol conservation of 3 monoclonal transformants of Ruxyn-pET-21a. Extract plasmid.

Jun 18 Send plasmid of Ruxyn-pET-21a transformant for sequencing.

Jun 20 Sequencing of Ruxyn-pET-21a correct.

Jul 19 Receive Ruxyn-9k-F/S primers. Dilute with dd$H_2O$ to 10uM.

Jul 20 (1) Temperature gradient PCR using Ruxyn-pET-21a as template and Ruxyn-9k-F/S as primers.

(2) Gel extract the PCR product.

(3) Digest extraction product using EcoRI and MluI.

Jul 21 Incubate surface display vector 9k strain.

Jul 22 (1) Extract plasmid from 9k strain.

(2) Water bath for Ruxyn digestion product from July 20 at 80℃ for 5min to inactivate restriction endonuclease.

(3) Ligate Ruxyn with vector 9k.

Jul 23 Transform Ruxyn-9k into DH5α competent cells.

Jul 24 Transform of Ruxyn-9k fails.

Jul 25 (1) PCR using Ruxyn-pET-21a as template and Ruxyn-9k-F/S as primers.

(2) Gel extract PCR product.

Jul 26 Ligate Ruxyn with pMD18-T vector. Transform ligation product into DH5α competent cells.

Jul 27 (1) Pick monoclones from transformation plate for streak cultivation on another plate.

(2) Pick monoclonal transformants from the plate for colony PCR.

Jul 28 Agarose gel electrophoresis of colony PCR shows positive. Pick 3 monoclonal transformants for incubation.

Jul 29 Conserve Ruxyn-pMD18-T strain, extract plasmid. Digest using EcoRI and MluI, correct.

Jul 30 Digest Ruxyn-pMD18-T plasmid and 9k vector using EcoRI and MluI. Gel extract the digested Ruxyn gene fragment and 9k vector fragment. Ligate both and transform into DH5α competent cells.

Jul 31 Pick monoclones for colony PCR, all results show positive. Pick 2 monoclones for incubation.

Aug 1 Conserve Ruxyn-9k strain, extract plasmid. Digest with EcoRI and MluI, correct.

Aug 2 Send Ruxyn-9k plasmids with both correct colony PCR and digestion for sequencing.

Aug 4 Sequencing correct.

**Standardization of *Pichia pastoris* secretory vector pPICZα**

May 20 Receive primers cel5A-EPS-F/S, dilute with $ddH_2O$ to 10uM.

May 21 (1) Temperature gradient PCR with cel5A-pMD 18-T as template and cel5A-EPS-F/S as primers to explore the optimal annealing temperature. Result of agarose gel electrophoresis shows that 60℃ is the optimum temperature.

(2) Incubate *Pichia pastoris* strains with secrete vector pPICZα in liquid LB medium.

May 22 Glycerol conservation of pPICZα strains. Extract plasmid.

May 24 PCR with cel5A-pMD 18-T as template and cel5A-EPS-F/S as primers. Gel extract the PCR products and name as cel5A-EPS.

May 26 (1) Use EcoRI and SpeI to digest both cel5A-EPS gel extraction product and pPICZα plasmid.

(2) Digestion of cel5A-EPS successful, extract. Digestion of pPICZα fails, repeat, fails again.

(3) Incubate pPICZα strains in liquid LB medium.

May 27 Extract pPICZα plasmid and digest again.

May 28 (1) Digestion of pPICZα successful, extract digestion product.

(2) Ligate cel5A-EPS digestion product of May 26 with pPICZα vector.

May 29 Transform cel5A-EPS-pPICZα ligation product. Transform fails, ligate again.

May 30 Re-transform cel5A-EPS-pPICZα ligation product.

May 31 (1) Pick monoclonal transformants for streak cultivation on another LB plate. Incubate 10h at 37℃.

(2) Pick 19 colonies. Colony PCR with cel5A-EPS-F/S as template.

(3) Pick colonies that showed positive in colony PCR and inoculate in 5ml liquid LB medium. Incubate at 37℃ overnight.

Jun 1 Glycerol conservation of transformant cel5A-EPS-pPICZα. Extract plasmid. Digest with EcoRI and PstI, correct.

Jun 4 Send cel5A-EPS-pPICZα transformants with the correct colony PCR and digestion for sequencing.

Jun 6 Sequencing of cel5A-EPS-pPICZα correct.

**Construction of Lignin peroxidase(LiP) BBa_K500000, Mn peroxidase(MnP) BBa_K500001, laccase BBa_K500002, Glyoxal oxidase(GLOX) BBa_K500003 secretory vectors**

Jul 17 Incubate strains with BBa_K500000, BBa_K500001, BBa_K500002, and BBa_K500003 genes repectively. Incubate strain with standardized cel5A-EPS-pPICZα.

Jul 18 (1) Conserve strains with BBa_K500000, BBa_K500001, BBa_K500002, BBa_K500003 and cel5A-EPS-pPICZα using glycerol. Extract plasmid.

(2) Digest BBa_K500000, BBa_K500001, BBa_K500002, BBa_K500003 and cel5A-EPS-pPICZα using EcoRI and PstI.

Jul 19 (1) Digestion of BBa_K500002 fails, others succeed. Gel extract digestion products.

(2) Ligate BBa_K500000, BBa_K500001, BBa_K500003 with pPICZα vector, respectively.

Jul 20 (1) Transform BBa_K500000-pPICZα, BBa_K500001-pPICZα, BBa_K500003-pPICZα into DH5α competent cells.

(2) Re-digest BBa_K500002 using EcoRI and PstI, fails again.

Jul 21 (1) Pick monoclonal transformants of BBa_K500000-pPICZα, BBa_K500001-pPICZα, BBa_K500003-pPICZα for streak cultivation on another plate.

(2) Re-incubate strain containing BBa_K500002-pSB1C3.

Jul 22 Extract plasmid from BBa_K500002-pSB1C3.

Jul 23 PCR using BBa_K500002-pSB1C3 as template and VR/VF2 as primers. Result shows that target band is only about 500bp, which is inconsistent with the size of the gene. Thus we assume that the vector is erroneous.

Jul 24 (1) Colony PCR of BBa_K500000-pPICZα, BBa_K500001-pPICZα, BBa_K500003-pPICZα transformants, all show positive results.

(2) Pick 4 transformants of BBa_K500000-pPICZα, BBa_K500001-pPICZα, BBa_K500003-pPICZα, respectively, and incubate.

(3) Send BBa_K500002-pSB1C3 for sequencing.

Jul 25 (1) Conserve strains containing BBa_K500000-pPICZα, BBa_K500001-pPICZα, BBa_K500003-pPICZα, extract plasmid.

(2) Digest BBa_K500000-pPICZα, BBa_K500001-pPICZα, BBa_K500003-pPICZα plasmids using EcoRI and PstI, all correct.

Jul 26 (1) Sequencing result of BBa_K500002-pSB1C3 shows that this plasmid is erroneous. Thus we give up using this gene.

(2) Send BBa_K500000-pPICZα, BBa_K500001-pPICZα, BBa_K500003-pPICZα for sequencing.

Jul 25 Sequencing of BBa_K500000-pPICZα, BBa_K500001-pPICZα, BBa_K500003-pPICZα all prove correct.

**Transformation of Lignin peroxidase(LiP) BBa_K500000, Mn peroxidase(MnP) BBa_K500001, laccase BBa_K500002, Glyoxal oxidase(GLOX) BBa_K500003 into *Pichia pastoris* and downstream assay**

Jul 28 Prepare medium and reagents for transformation.

Jul 30 Streak cultivation of GS115 yeast strain on YPD plate.

Aug 1 Incubate strains containing BBa_K500000-pPICZα, BBa_K500001-pPICZα, BBa_K500003-pPICZα.

Aug 2 Pick colonies of GS115 from streak plate. Inoculate into 5ml liquid YPD medium and incubate at 28℃.

Aug 3 (1) Linearize BBa_K500000-pPICZα, BBa_K500001-pPICZα, BBa_K500003-pPICZα plasmids: digest with SacI.

(2) Gel extract linearized plasmids.

Aug 4 Electro-transform BBa_K500000-pPICZα, BBa_K500001-pPICZα, BBa_K500003-pPICZ into *Pichia pastoris* GS115. Incubate at 28℃ for 2-3 days.

Aug 7 (1) A few colonies appear on BBa_K500000-pPICZα, BBa_K500001-pPICZα plates. Pick colonies for incubation.

(2) Electro-transformation of BBa_K500003-pPICZα fails.

(3) Prepare for another electro-transformation: inbucate BBa_K500000-pPICZα, BBa_K500001-pPICZα, BBa_K500003-pPICZα, pick colonies from GS115 plate and

incubate.

Aug 8 (1) Extract plasmids from strains containing BBa_K500000-pPICZα, BBa_K500001-pPICZα, BBa_K500003-pPICZα. Linearize using SacI. Extract linearized plasmid.

(2) Conserve electro-transformed yeasts containing BBa_K500000-pPICZαand BBa_K500001-pPICZα, extract genome. PCR with genome DNA as template, result shows positive.

Aug 9 Re-electrotransform BBa_K500000-pPICZα, BBa_K500001-pPICZα, BBa_K500003-pPICZα into GS115 yeasts.

Aug 12 Many BBa_K500000-pPICZα, BBa_K500001-pPICZα, BBa_K500003-pPICZαmonoclonal transformants appear. Screen using Zeocin resistance gradient screening methods.

Aug 15 Yeasts grow well on resistance gradient plate. Pick 4 colonies of each plate for incubation.

Aug 16 Conserve electro-transformed yeasts containing BBa_K500000-pPICZα, BBa_K500001-pPICZα, BBa_K500003-pPICZα, extract genome. PCR with genome DNA as template, all results are positive.

Aug 17 Incubate positive transformants of BBa_K500000-pPICZα, BBa_K500001-pPICZα, BBa_K500003-pPICZα genome PCR in 5ml liquid YPD.

Aug 18-23 Inoculate 1ml culture into 500ml conical flask containing 50ml BMGY. Cultivate at 28℃, 200r/min. Add 500ul 1% methanol every 24h, inductive cultivation for 120h.

Aug 24 (1) Determine enzyme activities of BBa_K500000-pPICZα, BBa_K500001-pPICZα, BBa_K500003-pPICZα. No notable enzyme activities.

(2) SDS-PAGE analysis of BBa_K500000-pPICZα, BBa_K500001-pPICZα, BBa_K500003-pPICZα. No specific bands occur, which might be due to low enzyme expression level.

Aug 25-28 Reincubate and ferment strains with BBa_K500000-pPICZα, BBa_K500001-pPICZα, BBa_K500003-pPICZα. SDS-PAGE analysis of supernatant, still no specific bands.

**Transformation of bglX-ZXFα, cel5A-9k, xyn-ZXFα, Ruxyn-9k into *Pichia pastoris* and downstream assay**

Aug 18 Incubate DH5α strains containing bglX-ZXFα, cel5A-9k, xyn-ZXFα, Ruxyn-9k.

Aug 19 Extract plasmid from the strains above.

Aug 22 (1) Linearize 222B-ZXFα and xyn–ZXFα using SacI. Linearize cel5A-9k and Ruxyn-9k using SalI. Gel extract linearized plasmid.

(2) Incubate strains containing pPICZαand 9k.

Aug 23 (1) Extract pPICZαand 9k, linearize using SacI and SalI, respectively. Gel extract linearized vectors.

(2) Electro-transform 222B-ZXFα, cel5A-9k, xyn –ZXFα, Ruxyn-9k and empty vectors pPICZαand 9k into *Pichia pastoris* GS115. Cultivate at 30℃ for 2-3 days.

Aug 26 Streak cultivate electrotransformed transformants of cel5A-9k, Ruxyn-9k and 9k on 2mg/ml G418 YPD, screen by resistance. Streak cultivate pPICZα on 100ug/ml Zecoin YPD, screen by resistance. Colonies of 222B-ZXFαand xyn–ZXFα are small, continue cultivation.

Aug 27 Pick colonies of 222B-ZXFαand xyn–ZXFα from transformation plate for incubation.

Aug 28 (1) Extract genome from yeasts containing 222B-ZXFαand xyn–ZXFα.
(2) Pick colonies of cel5A-9k, Ruxyn-9k and pPICZα from the plate for incubation.

Aug 29 (1) Extract genome of yeasts containing cel5A-9k, Ruxyn-9k and pPICZα.
(2) PCR 222B-ZXFα, cel5A-9k, xyn –ZXFα, Ruxyn-9k and pPICZα using genome as template, all results are positive.

Aug 30 (1) Incubate yeast transformants of cel5A-9k, Ruxyn-9k and 9k for incubation.
(2) 222B-ZXFαand xyn –ZXFα need to be re-electrotransformed. Transform 222B-ZXFαand xyn –ZXFα into DH5α.

Aug 31 (1) Inoculate 1ml culture of cel5A-9k, Ruxyn-9k, 9k, respectively, into 500ml conical flasks with 50ml BMGY. Cultivate at 28 ℃, 200 r/min, add 500ul 1% methanol every 24h. Inductive cultivation for 120h.
(2) Extract plasmids 222B-ZXFα and xyn –ZXFα.

Sept 1 Linearize 222B-ZXFα and xyn–ZXFαby SacI, ill-digested. Reincubate strains of 222B-ZXFα and xyn –ZXFα.

Sept 2 Extract 222B-ZXFα and xyn–ZXFα and linearize by SacI. Gel extract linearized vector and eletrotransform into GS115 and X-33. Cultivate at 30℃ for 2-3 days.

Sept 5 Many colonies occur on transformation plate. Screen high copies on high Zeocin resistence plate.

Sept 6 Pick colonies from high copy plates for incubation.

Sept 7 Extract yeast genome.

Sept 8 (1) PCR using yeast genome of 222B-ZXFα and xyn –ZXFα as template, with positive results.
(2) Determine enzyme activities of cel5A-9k and Ruxyn-9k fermentation fluids. No notable enzyme activity.

Sept 9 Incubate 222B-ZXFα, xyn–ZXFα and pPICZα yeasts using 5ml liquid YPD.

Sept 10 Inoculate 1ml culture into 500ml conical flask with 50ml BMGY. Incubate at 28 ℃, 200 r/min. Add 5ul 1% methanol every 24h. Inductive cultivation for 120h.

Sept 14 Use flow cytometry and fluorescence upright microscope to determine cel5A-9k, Ruxyn-9k and 9k fermentation fluids.

Sept 18 Incubate 222B-ZXFα, xyn–ZXFα, cel5A-9k, Ruxyn-9k, 9k and pPICZα strains using 5ml YPD.

Sept 19 (1) Inoculate 1ml culture of 222B-ZXFα, xyn –ZXFα, cel5A-9k, Ruxyn-9k, 9k and pPICZα strains into 500ml conical flasks with 50ml BMGY, respectively. Cultivate at 28℃,200 r/min and add 500ul 1% methanol every 24h. Inductive cultivation for 120h.
(2) Determine flow cytometry and fluorescence upright microscope to determine 222B-ZXFα, xyn –ZXFα and pPICZα fermentation fluids.

**Standardization of cel5A and Ruxyn**

Aug 30 (1) Receive Ruxyn-sta-F/S and cel5A-sta-F/S primers, dilute with $ddH_2O$ to 10uM.
(2) Incubate cel5A-9k and Ruxyn-9k strains.

Aug 31 Extract plasmids from cel5A-9k and Ruxyn-9k strains.

Sept 1 Temperature gradient PCR using cel5A-9k and Ruxyn-9k as template and cel5A-sta-F/S and Ruxyn-sta-F/S as corresponding primers, confirm Tm as 59℃.

Sept 2 PCR using cel5A-9k and Ruxyn-9k as template, gel extract target fragment.

Sept 3 Second PCR using gel extraction products of cel5A-9k and Ruxyn-9k as template and sp-sta-F/R as primers. Gel extract product and ligate with pMD18-T, transform into DH5α competent cells.

Sept 4 Yeast lawn appear on cel5A-9k and Ruxyn-9k transformation plates.

Sept 5 Ligate product of second gel extraction of cel5A-9k and Ruxyn-9k with pMD18-T. Transform into DH5α competent cells.

Sept 6 Lawns appear on transformation plates of cel5A-9k and Ruxyn-9k again. Pick several monoclones for colony PCR, all negative. Pick 4 monoclones of each and incubate.

Sept 7 (1) Conserve cel5A-9k and Ruxyn-9k, extract plasmid.

(2) Re-ligate gel extraction products with pMD18-T, transform into DH5α competent cells.

Sept 8 PCR and digest cel5A-9k and Ruxyn-9k from yesterday, all negative.

Sept 9 Many single colonies appear on cel5A-9k and Ruxyn-9k transformation plates. Results of colony PCR are all positive. Pick 4 colonies of positive PCR results respectively for incubation.

Sept 10 (1) Conserve the strains above and extract plasmid. Digestions by EcoRI and PstI are both positive.

(2) Incubate cel5A, Ruxyn and pSB1C3 vector with both correct PCR and digestion results.

Sept 11 Extract plasmid from strains of yesterday. Digest by EcoRI and PstI. Gel extract digestion products.

Sept 12 Ligate cel5A and Ruxyn with pSB1C3 respectively. Transform into DH5α competent cells.

Sept 13 Lawn grows on plate. Re-ligate and transform.

Sept 14 Same as yesterday. ☹

Sept 15 Transform Ruxyn-pSB1C3 and cel5A-pSB1C3.

Sept 16 Many colonies occur on transformation plate. Colony PCR results are all positive. Incubate.

Sept 17 Conserve and extract plasmid. Digestion results of EcoRI and PstI digestion are positive. Send for sequencing.

Sept 19 Sequencing of Ruxyn-pSB1C3 and cel5A-pSB1C3 are correct.

**Point mutation and standardization of xyn**

Aug 30 (1) Receive xyn-sta-F/S and xyn-mut-F/S primers, dilute with $ddH_2O$ to 10uM.

(2) Incubate with xyn–ZXFα.

Sept 1 Extract plasmid of xyn–ZXFα.

Sept 3 PCR respectively using xyn–ZXFα as template and xyn-sta-F/xyn-mut-S and xyn-mut-F/xyn-sta-S as primers. Gel extraction.

Sept 4 (1) Second PCR using gel extraction product of the first PCR and xyn-sta-F/S as primers. Gel extract target fragment.

(2) Third PCR using gel extraction product of the second PCR and xyn-sta-F/S as primers. Gel extract target fragment.

Sept 5 Plus A reaction. Gel extract, ligate with pMD18-T. Transform into DH5α competent cells.

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| Sept 6 | Lawns appear on transformation plate. Pick several colonies for colony PCR, all negative. Pick 4 monoclones for incubation. |
| Sept 7 | (1) Conserve xyn, extract plasmid.<br>(2) Re-ligate with pMD18-T, transform into DH5α competent cells. |
| Sept 8 | PCR and digest xyn plasmid from yesterday, both negative. |
| Sept 9 | Many single colonies occur on xyn transformation plate. Result of colony PCR is positive. Pick 7 strains with positive PCR result for incubation. |
| Sept 10 | (1) Conserve and extract plasmid. Digestion by EcoRI and PstI is positive.<br>(2) Incubate xyn and pSB1C3 vector with correct PCR and digestion results. |
| Sept 11 | Extract plasmid. Digest using EcoRI and PstI, gel extract digestion product. |
| Sept 12 | Ligate xyn with pSB1C3 vector, transform into DH5α competent cells. |
| Sept 13 | Lawns are spread all over culture plate. Re-ligate and transform. |
| Sept 14 | Same as yesterday. ☹ Religate. |
| Sept 15 | Transform xyn-pSB1C3. |
| Sept 16 | Still the same. ☹ Re-ligate and transform. |
| Sept 17 | Many colonies occur on transformation plate. Colony PCR results are all positive. Incubate. |
| Sept 18 | Extract plasmid. Digestion by EcoRI and PstI is positive. Send for sequencing. |
| Sept 20 | Sequencing correct. |