

1 **Characterization of the lysine forming saccharopine dehydrogenase**  
2 **LYS1 from filamentous fungi for plant food fermentation**

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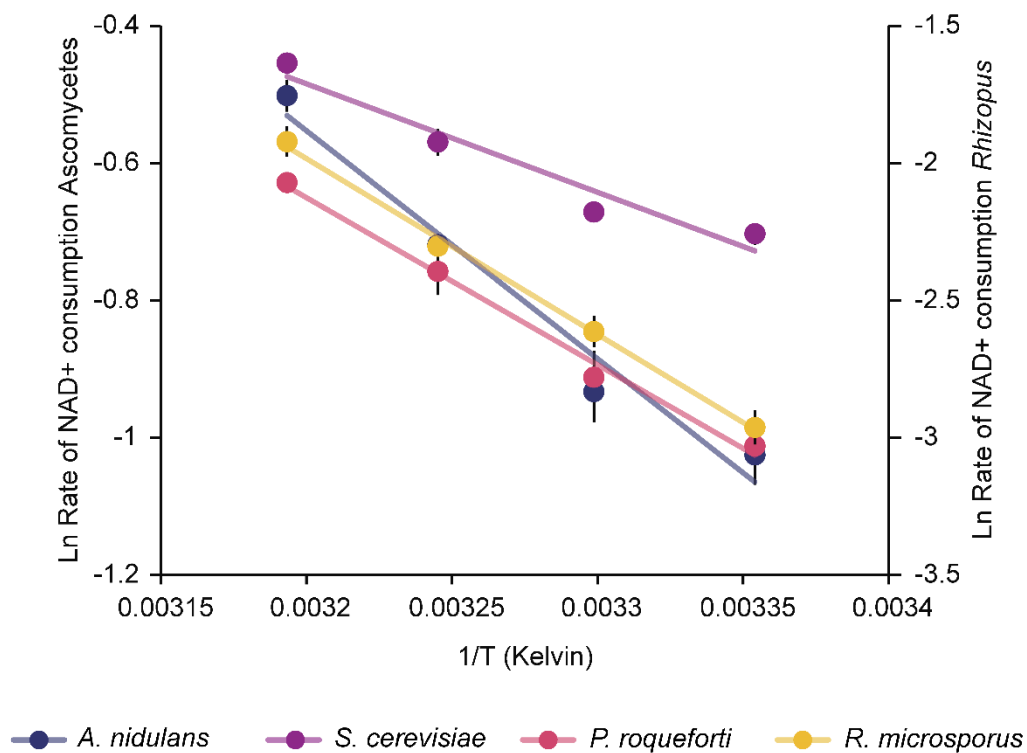
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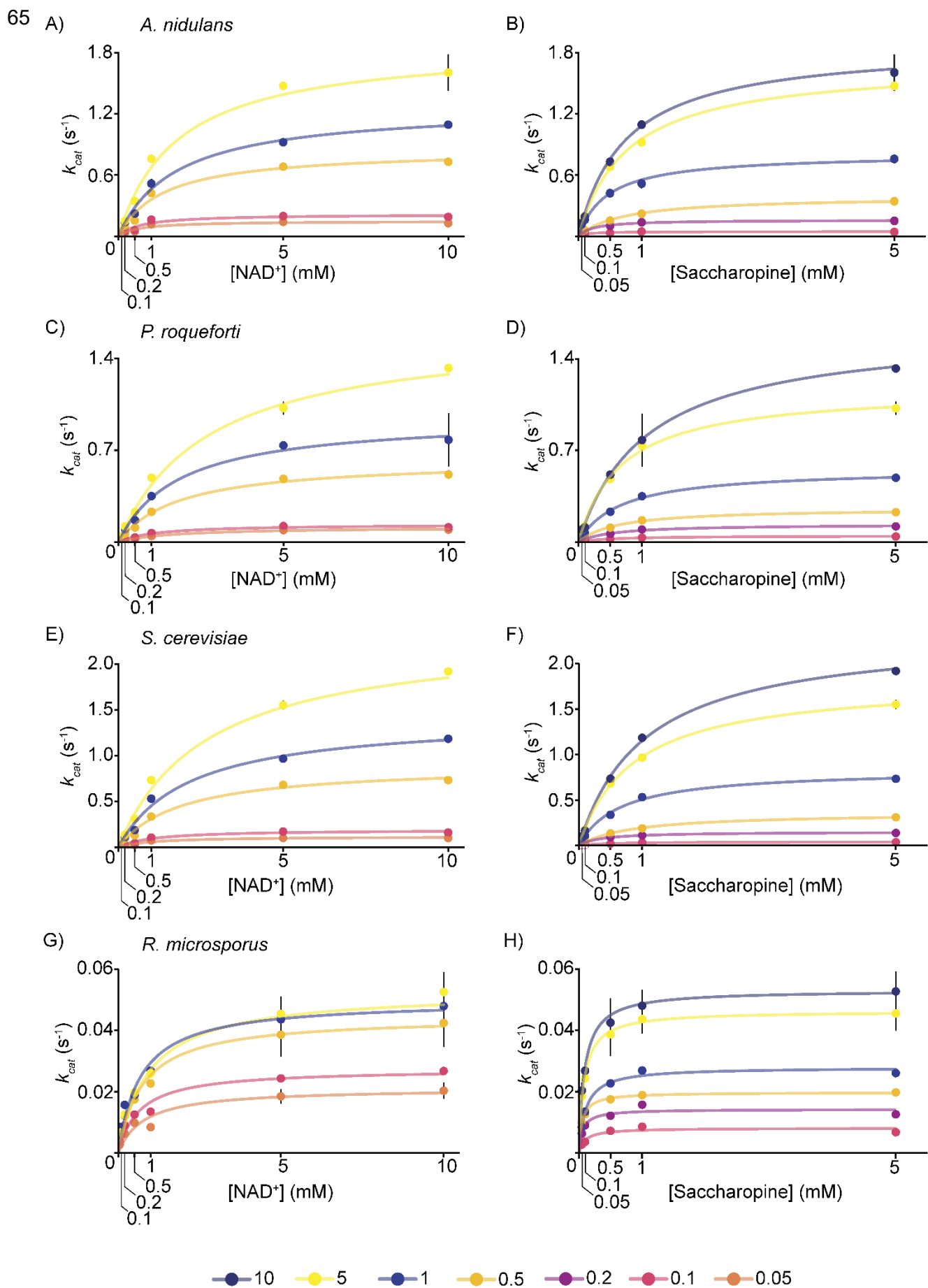
8 Supplementary material

Conservation:

Supplementary Figure S1. Multiple sequence alignment of fungal LYS1 proteins. Alignment of LYS1 from *S. cerevisiae* and 14 filamentous fungi. Red shading indicates identical residues blue shading indicates similar residues. Asterisks, colon signs, and periods indicate identical, strongly similar, and weakly similar alignment positions.

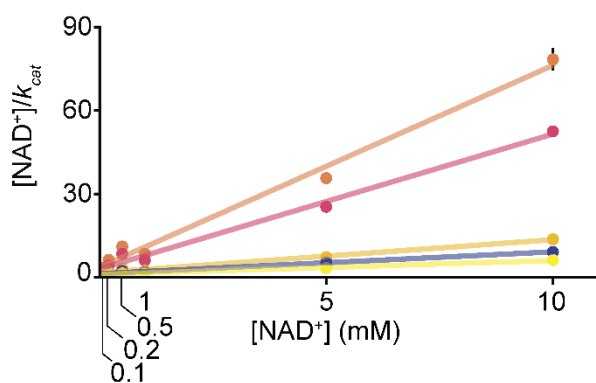


Supplementary Figure S2. Linearization of Arrhenius equation of the forward reaction catalyzed by LYS1 homologs. Natural logarithm of the NAD<sup>+</sup> consumption rate plotted against inverse temperature in Kelvin. Left axis applies to the Ascomycete homologs, right axis to *R. microsporus*. Data are mean  $\pm$  SD of triplicate measurements.

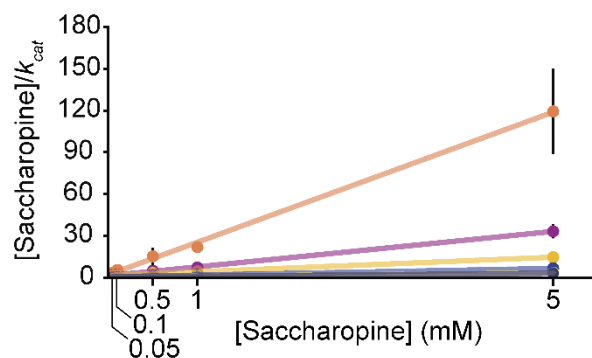


Supplementary Figure S3. Co-substrate dependence of the forward reaction catalyzed by LYS1 homologs. Turnover rates for (A, B) *A. nidulans*, (C, D) *P. roqueforti*, (E, F) *S. cerevisiae*, and (G, H) *R. microsporus*. Left panels show  $k_{\text{cat}}$  as a function of  $\text{NAD}^+$  concentration at fixed saccharopine concentrations; right panels show  $k_{\text{cat}}$  as a function of saccharopine concentration at fixed  $\text{NAD}^+$  concentrations. Curves represent nonlinear Michaelis-Menten fits. Data are mean  $\pm$  SD of triplicate measurements.

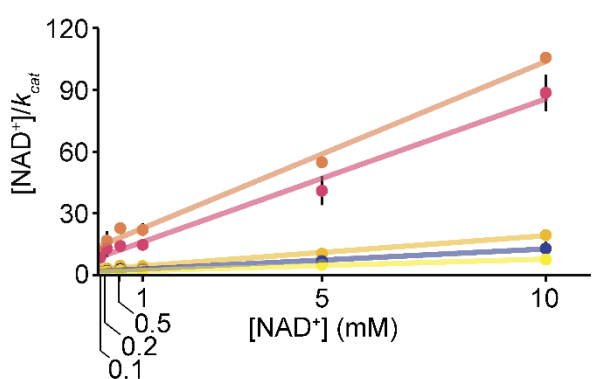
A) *A. nidulans*



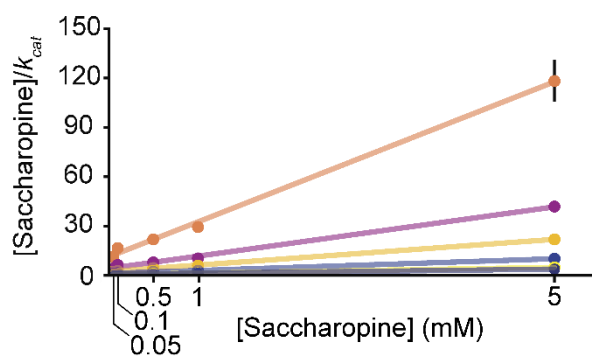
B)



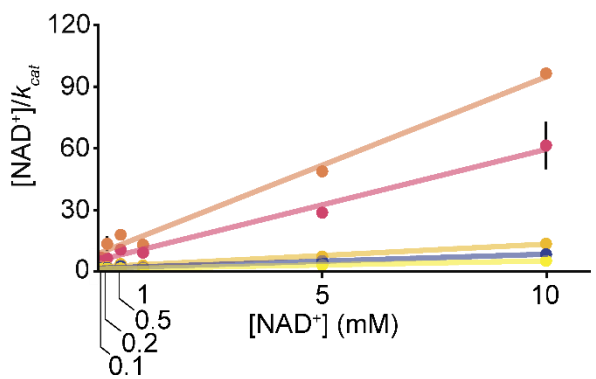
C) *P. roqueforti*



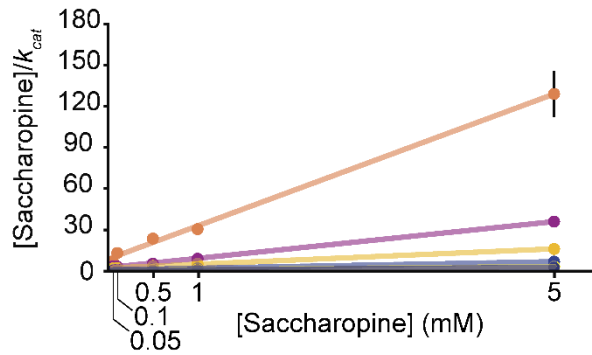
D)



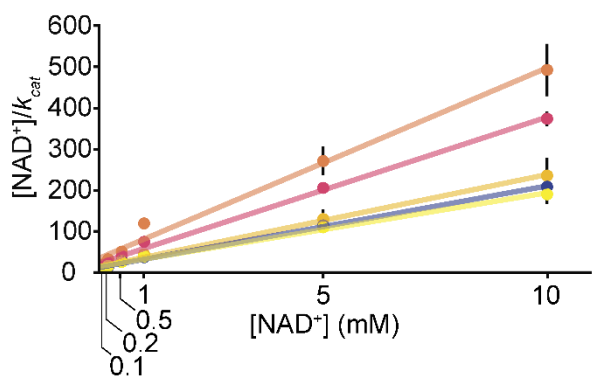
E) *S. cerevisiae*



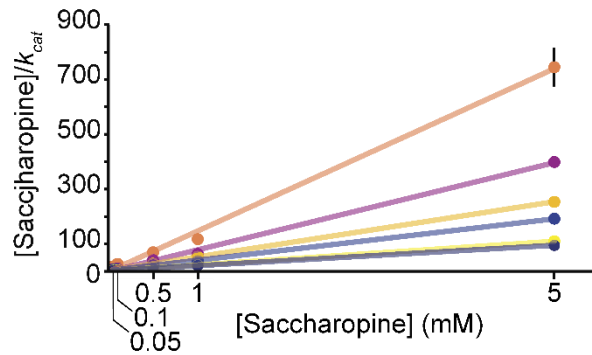
F)



G) *R. microsporus*

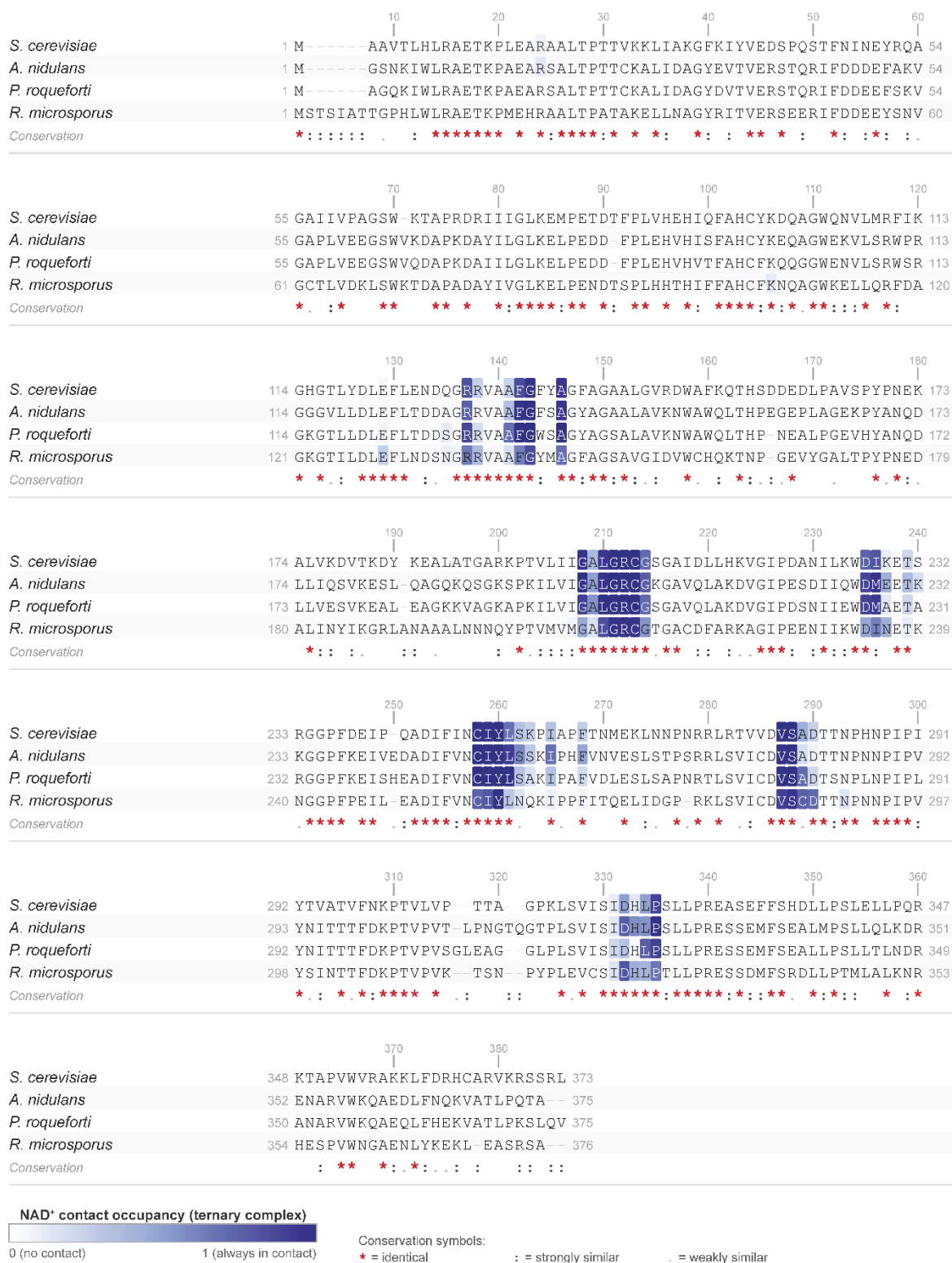


H)



● 10 ● 5 ● 1 ● 0.5 ● 0.2 ● 0.1 ● 0.05

Supplementary Figure S4. Hanes-Woolf analysis of the LYS1 substrate matrices. Linearized kinetics for (A, B) *A. nidulans*, (C, D) *P. roqueforti*, (E, F) *S. cerevisiae*, and (G, H) *R. microsporus*. Left panels show  $[\text{NAD}^+]/k_{\text{cat}}$  versus  $[\text{NAD}^+]$  at fixed saccharopine concentrations; right panels show  $[\text{saccharopine}]/k_{\text{cat}}$  versus  $[\text{saccharopine}]$  at fixed  $\text{NAD}^+$  concentrations. Data are mean  $\pm$  SD of triplicate measurements.



Supplementary Figure S5. NAD<sup>+</sup> contact occupancy mapped onto the alignment of experimentally characterized LYS1 homologs. Residues are shaded according to the fraction of ternary-complex simulation frames in contact with NAD<sup>+</sup>, from no contact to persistent contact. Occupancies were averaged across three independent 100-ns simulations.



Supplementary Table S1. LYS1 sequences and associated metadata used in the phylogenetic analysis. Species, strain, protein and genome accession numbers, sequence-retrieval method, and amino acid sequence are provided.

| Species name                    | Strain          | GenBank accession | Retrieval method  | Genome accession |
|---------------------------------|-----------------|-------------------|-------------------|------------------|
| <i>Saccharomyces cerevisiae</i> | 1278b           | X77362.1          | Literature        | GCA_000151485.1  |
| <i>Rhizopus arrhizus</i>        | Y5              | NA                | AUGUSTUS<br>BLAST | GCA_024220505.1  |
| <i>Rhizopus microsporus</i>     | ATCC 52813      | PHZ10661.1        | BLAST             | GCF_002708625.1  |
| <i>Rhizopus delemar</i>         | GL15            | KAG1453570.1      | BLAST             | GCA_011763585.1  |
| <i>Rhizopus stolonifer</i>      | B9770           | NA                | AUGUSTUS<br>BLAST | GCA_000697035.1  |
| <i>Aspergillus nidulans</i>     | FGSC A4         | XP_660477.2       | BLAST             | GCF_000011425.1  |
| <i>Aspergillus luchuensis</i>   | IFO 4308        | XP_041537339.1    | BLAST             | GCF_016861625.1  |
| <i>Aspergillus sojae</i>        | SMF134          | NA                | AUGUSTUS<br>BLAST | GCF_016861625.1  |
| <i>Aspergillus niger</i>        | CBS 513.88      | XP_001399884.1    | BLAST             | GCF_000002855.4  |
| <i>Aspergillus oryzae</i>       | RIB40           | XP_023090070.1    | BLAST             | GCF_000184455.2  |
| <i>Penicillium decumbens</i>    | NRRL 741        | OQD75955.1        | BLAST             | GCA_027569385.1  |
| <i>Penicillium chrysogenum</i>  | IBT 35668       | XP_056566197.1    | BLAST             | GCF_028827035.1  |
| <i>Penicillium verrucosum</i>   | IBT 35672       | KAJ5944149.1      | BLAST             | GCF_028828655.1  |
| <i>Penicillium roqueforti</i>   | LCP96 04111     | XP_038931514.1    | BLAST             | GCF_015533775.1  |
| <i>Penicillium camemberti</i>   | FM013=LCP0 6093 | CRL17805.1        | BLAST             | GCA_014839975.1  |

Supplementary Table S2. Pairwise amino acid sequence similarity between fungal LYS1 homologs. Values represent pairwise sequence similarity (%) for the 15 LYS1 homologs included in the multiple sequence alignment.

|                     | <i>Scerevisiae</i> | <i>Rarrhizus</i> | <i>Rmicrosporus</i> | <i>Rdeleamar</i> | <i>Rstolonifer</i> | <i>Anidulans</i> | <i>Aluchuensis</i> | <i>Asojae</i> | <i>Aniger</i> | <i>Aoryzae</i> | <i>Pdecumbens</i> | <i>Pchrysogenum</i> | <i>Pverrucosum</i> | <i>Proqueforti</i> | <i>Pcamemberti</i> |
|---------------------|--------------------|------------------|---------------------|------------------|--------------------|------------------|--------------------|---------------|---------------|----------------|-------------------|---------------------|--------------------|--------------------|--------------------|
| <i>Scerevisiae</i>  |                    | 50.4             | 50.1                | 50.1             | 50.5               | 54               | 55                 | 54.5          | 54.5          | 53.8           | 55.4              | 55.1                | 54.1               | 54.4               | 54.4               |
| <i>Rarrhizus</i>    | 50.4               |                  | 89.4                | 99.5             | 89.5               | 56.8             | 57.5               | 58.1          | 57.3          | 57.1           | 55.3              | 56.8                | 56.8               | 56.1               | 56.3               |
| <i>Rmicrosporus</i> | 50.1               | 89.4             |                     | 89.1             | 85.9               | 55.9             | 55.6               | 56.1          | 55.3          | 55.4           | 54.4              | 56                  | 56                 | 55.2               | 55.7               |
| <i>Rdeleamar</i>    | 50.1               | 99.5             | 89.1                |                  | 88.9               | 56.5             | 57.3               | 57.8          | 57            | 56.8           | 55                | 56.6                | 56.6               | 55.8               | 56.1               |
| <i>Rstolonifer</i>  | 50.5               | 89.5             | 85.9                | 88.9             |                    | 58.7             | 58.2               | 58.5          | 57.9          | 57.4           | 57.8              | 57.5                | 57.5               | 56.7               | 57                 |
| <i>Anidulans</i>    | 54                 | 56.8             | 55.9                | 56.5             | 58.7               |                  | 85.1               | 87.8          | 85.1          | 85.6           | 84.4              | 82                  | 81.7               | 80.7               | 81.7               |
| <i>Aluchuensis</i>  | 55                 | 57.5             | 55.6                | 57.3             | 58.2               | 85.1             |                    | 86.5          | 99.2          | 84.3           | 82.2              | 81.5                | 81                 | 80.2               | 81                 |
| <i>Asojae</i>       | 54.5               | 58.1             | 56.1                | 57.8             | 58.5               | 87.8             | 86.5               |               | 86.2          | 95.5           | 83.6              | 82.8                | 81.8               | 79.9               | 82.1               |
| <i>Aniger</i>       | 54.5               | 57.3             | 55.3                | 57               | 57.9               | 85.1             | 99.2               | 86.2          |               | 84.1           | 82.2              | 81.2                | 80.7               | 79.9               | 80.7               |
| <i>Aoryzae</i>      | 53.8               | 57.1             | 55.4                | 56.8             | 57.4               | 85.6             | 84.3               | 95.5          | 84.1          |                | 81.7              | 80.7                | 79.7               | 77.9               | 79.7               |
| <i>Pdecumbens</i>   | 55.4               | 55.3             | 54.4                | 55               | 57.8               | 84.4             | 82.2               | 83.6          | 82.2          | 81.7           |                   | 83.3                | 83.1               | 82                 | 83.1               |
| <i>Pchrysogenum</i> | 55.1               | 56.8             | 56                  | 56.6             | 57.5               | 82               | 81.5               | 82.8          | 81.2          | 80.7           | 83.3              |                     | 97.9               | 94.4               | 97.6               |
| <i>Pverrucosum</i>  | 54.1               | 56.8             | 56                  | 56.6             | 57.5               | 81.7             | 81                 | 81.8          | 80.7          | 79.7           | 83.1              | 97.9                |                    | 96                 | 99.2               |
| <i>Proqueforti</i>  | 54.4               | 56.1             | 55.2                | 55.8             | 56.7               | 80.7             | 80.2               | 79.9          | 79.9          | 77.9           | 82                | 94.4                | 96                 |                    | 96                 |
| <i>Pcamemberti</i>  | 54.4               | 56.3             | 55.7                | 56.1             | 57                 | 81.7             | 81                 | 82.1          | 80.7          | 79.7           | 83.1              | 97.6                | 99.2               | 96                 |                    |

Supplementary Table S3. Active site flexibility of LYS1 homologs under different simulated ligand conditions. Mean active site C $\alpha$  RMSF and standard deviation (nm) from triplicate molecular dynamics simulations of the apo, NAD<sup>+</sup>-bound, saccharopine-bound, and ternary complexes.  $\Delta$ RMSF was calculated as the ternary complex RMSF minus apo RMSF.

| Species                      | Condition               | Average | Standard Deviation |
|------------------------------|-------------------------|---------|--------------------|
| <b><i>S. cerevisiae</i></b>  | NAD <sup>+</sup> -bound | 0.119   | 0.003              |
|                              | Apo enzyme              | 0.129   | 0.019              |
|                              | Ternary complex         | 0.123   | 0.016              |
|                              | Saccharopine-bound      | 0.110   | 0.025              |
|                              | $\Delta$ RMSF           | -0.006  | 0.025              |
| <b><i>A. nidulans</i></b>    | NAD <sup>+</sup> -bound | 0.163   | 0.026              |
|                              | Apo enzyme              | 0.171   | 0.040              |
|                              | Ternary complex         | 0.118   | 0.012              |
|                              | Saccharopine-bound      | 0.140   | 0.036              |
|                              | $\Delta$ RMSF           | -0.053  | 0.042              |
| <b><i>P. roqueforti</i></b>  | NAD <sup>+</sup> -bound | 0.113   | 0.007              |
|                              | Apo enzyme              | 0.127   | 0.007              |
|                              | Ternary complex         | 0.097   | 0.012              |
|                              | Saccharopine-bound      | 0.150   | 0.030              |
|                              | $\Delta$ RMSF           | -0.031  | 0.014              |
| <b><i>R. microsporus</i></b> | NAD <sup>+</sup> -bound | 0.143   | 0.012              |
|                              | Apo enzyme              | 0.150   | 0.003              |
|                              | Ternary complex         | 0.121   | 0.006              |
|                              | Saccharopine-bound      | 0.106   | 0.004              |
|                              | $\Delta$ RMSF           | -0.029  | 0.007              |

Supplementary Table S4. Residue-ligand contact features contributing to the principal components of LYS1 ternary complex contact occupancy. The 20 highest-ranked contact features for PC1 and PC2 are listed with loading direction, ligand and alignment position, loading value, absolute loading, and rank.

| PC  | Direction | Contact and alignment position | Value   | Absolute value | Rank within PC |
|-----|-----------|--------------------------------|---------|----------------|----------------|
| PC1 | Positive  | NAD_c141                       | 0.2722  | 0.2722         | 1              |
| PC1 | Positive  | SAC_c304                       | 0.2648  | 0.2648         | 2              |
| PC1 | Positive  | SAC_c331                       | 0.2647  | 0.2647         | 3              |
| PC1 | Positive  | SAC_c334                       | 0.2632  | 0.2632         | 4              |
| PC1 | Positive  | SAC_c303                       | 0.2497  | 0.2497         | 5              |
| PC1 | Positive  | SAC_c289                       | 0.2325  | 0.2325         | 6              |
| PC1 | Positive  | SAC_c129                       | 0.2225  | 0.2225         | 7              |
| PC1 | Positive  | SAC_c106                       | 0.2185  | 0.2185         | 8              |
| PC1 | Positive  | SAC_c290                       | 0.217   | 0.217          | 9              |
| PC1 | Positive  | SAC_c292                       | 0.2113  | 0.2113         | 10             |
| PC1 | Negative  | SAC_c19                        | -0.1952 | 0.1952         | 11             |
| PC1 | Negative  | NAD_c207                       | -0.1832 | 0.1832         | 12             |
| PC1 | Negative  | NAD_c333                       | -0.1675 | 0.1675         | 13             |
| PC1 | Negative  | SAC_c22                        | -0.1636 | 0.1636         | 14             |
| PC1 | Negative  | NAD_c290                       | -0.1518 | 0.1518         | 15             |
| PC1 | Negative  | NAD_c332                       | -0.1332 | 0.1332         | 16             |
| PC1 | Negative  | NAD_c106                       | -0.124  | 0.124          | 17             |
| PC1 | Negative  | NAD_c24                        | -0.0788 | 0.0788         | 18             |
| PC1 | Negative  | NAD_c240                       | -0.0547 | 0.0547         | 19             |
| PC1 | Negative  | NAD_c268                       | 0.0053  | 0.0053         | 20             |
| PC2 | Positive  | NAD_c265                       | 0.288   | 0.288          | 1              |
| PC2 | Positive  | NAD_c240                       | 0.2848  | 0.2848         | 2              |
| PC2 | Positive  | NAD_c262                       | 0.2822  | 0.2822         | 3              |
| PC2 | Positive  | NAD_c268                       | 0.2741  | 0.2741         | 4              |
| PC2 | Negative  | NAD_c289                       | -0.2734 | 0.2734         | 5              |
| PC2 | Positive  | NAD_c24                        | 0.2725  | 0.2725         | 6              |
| PC2 | Negative  | SAC_c291                       | -0.2452 | 0.2452         | 7              |
| PC2 | Negative  | NAD_c290                       | -0.2203 | 0.2203         | 8              |
| PC2 | Positive  | NAD_c261                       | 0.2139  | 0.2139         | 9              |
| PC2 | Negative  | NAD_c207                       | -0.2083 | 0.2083         | 10             |
| PC2 | Positive  | SAC_c101                       | 0.2065  | 0.2065         | 11             |
| PC2 | Positive  | SAC_c141                       | 0.1764  | 0.1764         | 12             |
| PC2 | Positive  | NAD_c208                       | 0.1752  | 0.1752         | 13             |
| PC2 | Negative  | SAC_c22                        | -0.163  | 0.163          | 14             |
| PC2 | Negative  | NAD_c106                       | -0.16   | 0.16           | 15             |
| PC2 | Negative  | SAC_c106                       | -0.1499 | 0.1499         | 16             |
| PC2 | Negative  | SAC_c103                       | -0.1481 | 0.1481         | 17             |
| PC2 | Positive  | SAC_c346                       | 0.1435  | 0.1435         | 18             |
| PC2 | Negative  | SAC_c335                       | -0.1316 | 0.1316         | 19             |
| PC2 | Negative  | SAC_c292                       | -0.1207 | 0.1207         | 20             |

Supplementary Table S5. Count of residues of LYS1 in contact with NAD<sup>+</sup> or saccharopine during molecular dynamics simulations. Residue counts are reported at contact-occupancy thresholds greater than 0%, 20%, and 50% for single ligand and ternary complex simulations of each homolog.

| Species                      | Condition               | Ligand           | Residues<br>>0% contact | Residues<br>>20%<br>contact | Residues<br>>50%<br>contact |
|------------------------------|-------------------------|------------------|-------------------------|-----------------------------|-----------------------------|
| <b><i>A. nidulans</i></b>    | NAD <sup>+</sup> -bound | NAD <sup>+</sup> | 47                      | 33                          | 23                          |
|                              | Sacc-bound              | Saccharopine     | 38                      | 21                          | 12                          |
|                              | Ternary                 | NAD <sup>+</sup> | 41                      | 30                          | 24                          |
|                              | Ternary                 | Saccharopine     | 25                      | 15                          | 11                          |
| <b><i>P. roqueforti</i></b>  | NAD <sup>+</sup> -bound | NAD <sup>+</sup> | 42                      | 29                          | 23                          |
|                              | Sacc-bound              | Saccharopine     | 49                      | 16                          | 5                           |
|                              | Ternary                 | NAD <sup>+</sup> | 39                      | 29                          | 23                          |
|                              | Ternary                 | Saccharopine     | 30                      | 20                          | 18                          |
| <b><i>R. microsporus</i></b> | NAD <sup>+</sup> -bound | NAD <sup>+</sup> | 45                      | 29                          | 17                          |
|                              | Sacc-bound              | Saccharopine     | 34                      | 22                          | 18                          |
|                              | Ternary                 | NAD <sup>+</sup> | 46                      | 27                          | 19                          |
|                              | Ternary                 | Saccharopine     | 28                      | 21                          | 10                          |
| <b><i>S. cerevisiae</i></b>  | NAD <sup>+</sup> -bound | NAD <sup>+</sup> | 50                      | 34                          | 17                          |
|                              | Sacc-bound              | Saccharopine     | 34                      | 20                          | 13                          |
|                              | Ternary                 | NAD <sup>+</sup> | 40                      | 31                          | 21                          |
|                              | Ternary                 | Saccharopine     | 33                      | 18                          | 9                           |