Supplementary material – gene and protein sequences

Figure. S1 Sequences of genes optimized for expression in *Escherichia coli* and the corresponding nitrilases from *Trametes versicolor* (A), *Armillaria gallica* (B) and *Stereum hirsutum* (C) with restriction sites underlined.
Supplementary data - Nitrilase sequences in *Agaricomycotina*

**Table S1.** List of nitrilase sequences in the species of *Agaricomycotina* according to GenBank and their classification into clades

| Species                   | GenBank accession no. | Length (amino acids) | Clade | Closest characterized homologue Identity (%) / (Cover, %) |
|---------------------------|-----------------------|----------------------|-------|---------------------------------------------------------|
| *Agaricus bisporus v bisporus* |                       |                      |       |                                                         |
| Apiotrichum porosum       | XP_006462086.1         | 339                  |       |                                                         |
|                           | XP_028472322.1         | 352                  |       |                                                         |
|                           | XP_028475380.1         | 366                  |       |                                                         |
| *Armillaria gallica*      | PBL01211.1 (NitAg)     | 356                  | 2     | NitTv1 74 (96)                                          |
|                           | PBL01250.1             | 314                  | 1     | NitTv1 74 (96)                                          |
| *Armillaria ostoyae*      |                       |                      |       |                                                         |
|                           | PBK95426.1             | 336                  | 2     | NitAg 70 (93)                                           |
|                           | PBK82882.1             | 355                  | 2     | NitAg 83 (96)                                           |
|                           | SJL05523.1             | 356                  | 2     | NitAg 97 (100)                                          |
|                           | PBK74191.1             | 355                  | 2     | NitAg 84 (96)                                           |
|                           | SJL05484.1             | 314                  |       | NitTv1 74 (97)                                          |
|                           | PBK77460.1             | 314                  |       | NitTv1 73 (97)                                          |
| *Auricularia subglabra*   | EJD42068.1 (NitAd)*    | 331                  | 3     | NitTv1 69 (96)                                          |
|                           | EJD55093.1             | 318                  | 1     | NitTv1 69 (96)                                          |
|                           | EJD51184.1             | 365                  | CynH  | NitSh 85 (97)                                           |
|                           | EJD51215.1             | 346                  | CynH  | NitSh 85 (100)                                          |
|                           | EJD54336.1             | 365                  | CynH  | NitSh 75 (94)                                           |
| *Bondarzewia mesenterica* | THH19653.1             |                      |       | NitAg 56 (97)                                           |
| *Botryobasidium botryosum*|                       |                      |       |                                                         |
| *Calocera viscosa*        | KDO8029.1              | 328                  | 1     | NitTv1 71 (92)                                          |
| *Cenangiophora puteana*   | KZO96291.1             | 310                  | 1     | NitTv1 55 (98)                                          |
| *Cryptococcus amylolentus*|                       |                      |       |                                                         |
| *Cryptococcus neoformans* |                       |                      |       |                                                         |
|                           | XP_018995183.1         | 342                  |       |                                                         |
|                           | OXB39643.1             | 366                  |       |                                                         |
|                           | OWZ73108.1             | 309                  |       |                                                         |
| Species                                      | Accession     | Start | End | NitTV1 | NitAg | NitSh |
|----------------------------------------------|---------------|-------|-----|--------|-------|-------|
| *Cutaneotrichosporon oleaginosum*            | XP_012046763.1| 309   |     |        |       |       |
| *Cutaneotrichosporon oleaginosum*            | XP_018275718.1| 333   |     |        |       |       |
| *Cylindrobasidium torrendii*                 | KIY65145.1    | 311   | 1   |        |       | NitTv1 67 (95) |
| *Dacryopinax primogenitus*                   | EJT97776.1    | 340   | 1   | NitTv1 36 (91) |
| *Dacryopinax primogenitus*                   | THV07691.1    | 316   | 1   | NitTv1 74 (97) |
| *Dendrothele bispora*                        | THV07653.1    | 367   | 2   | NitAg 81 (94) |
| *Dentipellis fragilis*                       | THU83280.1    | 340   |     |        |       |       |
| *Dichomitus squalens*                        | XP_007360414.1| 319   | 1   | NitTv1 87 (100) |
| *Dichomitus squalens*                        | KZW02628.1    | 313   | 1   | NitTv1 70 (98) |
| *Exidia glandulosa*                          | KZV92691.1    | 366   | CynH | NitTv1 82 (100) |
| *Fibularhizoctonia sp.*                      | KZP15294.1    | 371   |     |        |       |       |
| *Fistulina hepatica*                         | KIY50558.1    | 331   | 1   | NitTv1 68 (96) |
| *Fomitopsis mediterranea*                    | XP_007265585.1| 318   | 1   | NitTv1 72 (97) |
| *Ganoderma sinense*                          | PIL31680.1    | 333   | 1   | NitTv1 83 (98) |
| *Gelatoporia subvermispora*                  | EMD41986.1    | 317   | 1   | NitTv1 77 (97) |
| *Grifola frondosa*                           | OBZ78624.1    | 295   | 1   | NitTv1 76 (97) |
| *Gymnoporus luxurians*                       | KJK70890.1    | 316   | 1   | NitTv1 75 (96) |
| *Heliocybe alpestrae*                        | TFK54302.1    | 322   |     |        |       |       |
| *Hiricium alpestrae*                         | TFY81337.1    | 2     |     | NitAg 70 (96) |
| *Hiricium alpestrae*                         | TFY75083.1    | 2     |     | NitAg 63 (91) |
| *Heterobasidion irregular*                   | XP_009540410.1| 315   | 1   | NitTv1 74 (97) |
| *Hydnoderus pinastri*                        | XP_009553498.1| 353   | 2   | NitAg 72 (96) |
| *Hyphsizygus marmoreus*                      | KIJI68545.1   | 316   | 1   | NitTv1 74 (96) |
| *Jappia argillacea*                          | KDQ63493.1    | 342   | 1   | NitTv1 73 (98) |
| *Kockovskella imperatae*                     | XP_021872624.1| 332   |     |        |       |       |
| *Kwoniella bestiolae*                        | XP_019049889.1| 346   |     |        |       |       |
| *Kwoniella dejecticola*                      | XP_018266860.1| 344   |     |        |       |       |
| *Kwoniella heveamensis*                      | OCF37843.1    | 342   |     |        |       |       |
| Genus                  | Accession          | Query Length | Nitrogenase Activity |
|------------------------|--------------------|--------------|----------------------|
| *Kwoniella magroviensis* | OCF30510.1         | 358          | NitAg 80 (97)        |
|                        | OCF41321.1         | 308          |                      |
|                        | OCF35479.1         | 326          |                      |
|                        | XP_018999060.1     | 360          |                      |
|                        | XP_018999159.1     | 351          |                      |
|                        | OCF54328.1         | 376          |                      |
|                        | OCF58370.1         | 360          |                      |
|                        | XP_019000820.1     | 350          |                      |
|                        | OCF62338.1         | 327          |                      |
|                        | XP_019009856.1     | 345          |                      |
| *Kwoniella pini*       |                    |              |                      |
| *Lentinula edodes*     | GAW06039.1         | 360          | 2                    |
|                        |                    |              | NitTv1 80 (97)       |
| *Lentinus tigrinus*    | RPD64846.1         | 320          | 1                    |
|                        | ESK97990.1         | 315          | 1                    |
|                        | ESK97956.1         | 358          | 2                    |
|                        |                    |              | NitAg 81 (97)        |
| *Moniliophthora rorera*|                    |              |                      |
| *Mycena chlorophos*    | GAT44080.1         | 351          | 2                    |
|                        |                    |              | NitAg 72 (98)        |
| *Naematelia encephala*| GAT47984.1         | 331          |                      |
|                        | GAT55790.1         | 320          |                      |
| *Neolentinus lepideus* | ORY27096.1         | 336          |                      |
|                        | ORY35932.1         | 358          |                      |
| *Obba rivulosa*        | OCH94772.1         | 317          | 1                    |
| *Paxillus involutus*   | KIJ21834.1         | 315          | 1                    |
| *Peniophora sp.*       | KZV75469.1         | 308          | 1                    |
| *Phanerochaete carnosa*|                    |              |                      |
|                        | XP_007390669.1     | 318          | 1                    |
|                        | XP_007401608.1     | 306          | 1                    |
| *Phellinidium pouzarii*| THH09479.1         | 339          | 1                    |
| *Phlebiopsis gigantea* | KIP12511.1         | 317          | 1                    |
| *Pleurotus ostreatus*  | KDQ30886.1         | 322          | 1                    |
|                        | KDQ32741.1         | 304          | 2                    |
|                        | KDQ30928.1         | 305          | 2                    |
|                        |                    |              | NitAg 76 (85)        |
| Species                              | Accession | Length | Ident | Validation |
|--------------------------------------|-----------|--------|-------|------------|
| **Plicaturopsis crispa**              | KII93875.1| 312    | 1     | NitTv1 78 (97) |
|                                     | KII92786.1| 359    | 2     | NitAg 68 (98) |
| **Pluteus cervinus**                 | TFK64162.1| 334    |       |            |
| **Polyporus arcularius**             | TFK62976.1| 321    | 1     | NitTv1 84 (99) |
| **Polyporus brumalis**               | RDX50451.1| 321    | 1     | NitTv1 84 (99) |
| **Punctularia strigoszonata**        | XP_007379045.1| 314 | 1     | NitTv1 68 (95) |
| **Rhizoctonia solani**               | CUA68841.1| 320    | 1     | NitTv1 65 (98) |
| **Saitozyma podzolica**              | RSH94106.1| 387    |       |            |
| **Sanghuangporus baumii**            | OCB88554.1| 319    | 1     | NitTv1 71 (95) |
| **Sarzhyngoporus youkii**            | XP_003037202.1| 317 | 1     | NitTv1 71 (96) |
| **Schizophyllum commune**            | XP_00037202.1| 317 | 1     | NitTv1 71 (96) |
| **Schizopora paradoxa**              | KLO14490.1| 325    | 1     | NitTv1 62 (97) |
| **Serpula lacrymans**                | EGO05225.1| 311    | 1     | NitTv1 71 (97) |
| **Sistotremastrum niveocremeum**     | KZS96174.1| 311    | 1     | NitTv1 70 (98) |
| **Sistotremastrum suecicum**         | KZT44489.1| 311    | 1     | NitTv1 70 (98) |
| **Sphaerotholus stellatus**          | KIJ45626.1| 317    | 1     | NitTv1 68 (97) |
| **Stereum ochraceum**                | TCD67848.1| 313    |       |            |
| **Stereum hirsutum**                 | XP_007307917.1 (NitSh) | 371 | CynH | – |
| **Stereum hirsutum**                 | XP_007307917.1 | 371 | CynH | – |
| **Suillus luteus**                   | KIK46442.1| 316    | 1     | NitTv1 71 (98) |
| **Termitomyces sp.**                 | KNZ75542.1| 314    | 1     | NitTv1 69 (98) |
| Species                      | Accession      | Length | Identity | Name          |
|------------------------------|----------------|--------|----------|---------------|
| *Trametes cinnaburina*       | CDO73495.1     | 289    | 1        | NitTv1 83 (81) |
| *Trametes coccinea*          | OSC99476.1     | 338    | 1        | NitTv1 91 (98) |
| *Trametes pubescens*         | OJT10100.1     | 339    | 1        | NitTv1 88 (100) |
| *Trametes versicolor*        | XP_008032838.1 | 320    | 1        | –             |
| *Trichosporon asahii*        | EKD01791.1     | 386    |          |               |
| *Xanthophyllomyces dendrorhous* | CDZ98326.1     | 319    |          |               |
|                              | CED82572.1     | 329    |          |               |

*a* synonym: *Armillaria solidipes*

*b* NitAd from *Auricularia delicata*, re-classified *Auricularia subglabra*

*c* synonym: *Sanghuangporus baumii*

The enzymes overproduced in *E. coli* are marked in bold. NitAg, NitSh and NitTv1 were produced in this study, NitAd in the previous study (ref. [16] in the main manuscript). Proteins with ≥99% amino acid sequence identity, which occur in the same species, were discarded.

Sequences in blue do not belong to clade 1 or 2 or CynHs.
## Supplementary data–Molecular modeling

**Table S2.** Normalized formation of HB during stable period of molecular docking (5–10 ns) in NitTv1 and NitAg nitrilase–fumaronitrile (FN) complexes.

| Complex   | Interacting residues | K133-FN | K133-E46 | K133-E140 | C178-E46 | V203-E46 | V203-C178 |
|-----------|----------------------|---------|----------|-----------|----------|----------|-----------|
| NitTv1-FN | Formation of HB      | 0.92    | 0.965    | 0.915     | 0        | 0.78     | 0.9       |
| NitAg-FN  | Formation of HB      | 0.765   | 0.95     | 0.815     | 0.265    | 0        | 0.025     |
| NitSh-FN  | Formation of HB      | 0.71    | 0.91     | 0.77      | 0        | 0        | 0         |
Figure. S2. Surface representation of the active sites of nitrilases with docked ligands. (A) NitAg with fumaronitrile (FN). The orientation of 3-phenylpropionitrile (3-PPN) and β-cyano alanine (β-CA) is made from structure alignment with NitTv1 complexed with corresponding ligands. (B) NitTv1 with FN, 3-PPN and β-CA. (C) Alignment of NitTv1 (blue), NitAg (red), NitSh (magenta) and 3wuy (green). W197 and F200 in NitAg are shown by red sticks, and the surface area occupied by them is marked in a red semitransparent colour. Part of the enzymes is hidden for clarity. (D) NitSh with FN, β-CA and 2-cyanopyridine, the orientation of which corresponds to complexes with NitSh. 3-PPN orientation corresponds to its position in NitTv1 complex.
Supplementary data–Product characterization

Products of fumaronitrile transformations by nitrilase NitTv1

To obtain samples for LC-MS, transformation of fumaronitrile (FN) was carried out using *Escherichia coli* whole cells carrying nitrilase (NLase) NitTv1 (dry cell weight 0.3 g/L) and 25 mM substrate in 50 mM Tris/HCl buffer, pH 8.0, with 150 mM NaCl (total volume 0.5 mL). The reaction proceeded at 30 °C and shaking (850 rpm) for 10 min. The reaction was terminated by adding 0.05 mL 2M HCl and the cells were removed by centrifugation. The supernatant was diluted with mobile phase (1:50) and analyzed by LC-MS (see Fig. S3 for the chromatogram and figure legend for m/z (ESI) data).

![HPLC chromatogram](chart.png)

Figure S3. HPLC of the products obtained from fumaronitrile (1a) using nitrilase NitTv1. Separations conditions: ACE 5 C8 (250 × 4 mm) column, mobile phase 10% acetonitrile in water, isocratic, flow rate 0.4 mL/min, 34 °C. m/z (ESI): 1b: [M-H]- calculated for C₄H₇NO₂ 96.0, found 96; [M+NO₃]- calculated
for C₇H₅N₂O₅ 159.1, found 159; 1c: [M+HCOO]- calculated for C₅H₅N₂O₃ 141.1, found 141; 1d: [M+Cl]- calculated for C₄H₅ClNO₃ 150.0, found 150. The minor product with RT = 5.313 is fumaric acid: [M+HCOO]- calculated for C₅H₇O₆ 161.1, found 161.

To obtain products for NMR (Figs S4 and S5), the conditions of the transformation of FN were modified (dry cell weight 0.6 g/L, reaction time 60 min, total volume 50 mL). After removing the cells by centrifugation, the supernatant was extracted with ethylacetate at pH 8 (pH of the reaction mixture) and then at pH 2 (adjusted with 2M HCl). The organic fractions from each extraction were pooled, dried with Na₂SO₄ and filtered, and the solvent was removed at reduced pressure.

The product extracted at pH 8 contained 1c as the major product (isolated yield 50%; 61 mg).

Figure S4. The detail of ¹H NMR spectrum of the product extracted at pH 8 (399.87 MHz, DMSO, 30 °C) with major compound 1c
NMR data: 1c - $^1$H NMR (399.87 MHz, DMSO, 30 °C): 6.479 (1H, d, $J = 16.3$ Hz, CH), 6.969 (1H, d, $J = 16.3$ Hz, CH), 7.608 (1H, br s, NH$_2$-u), 7.883 (1H, br s, NH$_2$-u); $^{13}$C NMR (100.55 MHz, DMSO, 30 °C): 108.65 (CH), 117.07 (CN), 144.05 (CH), 163.20 (CO).

The product extracted at pH 2.5 (isolated yield 17%; 20 mg) contained 1b as the major product (62% of total product). Compounds 1c, 1d, fumaric acid and its diamide were minor products with ca. 10%, 16%, 2% and 10% of the total product.

Figure S5. The detail of $^1$H NMR spectrum of the product extracted at pH 2.5 (399.87 MHz, DMSO, 30 °C) with major compound 1b

NMR data: 1b - $^1$H NMR (399.87 MHz, DMSO, 30 °C): 6.590 (1H, d, $J = 16.4$ Hz, CH), 6.904 (1H, d, $J = 16.4$ Hz, CH), OH not detected; $^{13}$C NMR (100.55 MHz, DMSO, 30 °C): 111.66 (CH), 116.62 (CN), 142.77 (CH), 164.81 (CO); 1d - $^1$H NMR (399.87 MHz, DMSO, 30 °C): 6.504 (1H, d, $J = 15.6$ Hz, CH), 6.880 (1H, d, $J = 15.6$ Hz, CH), NH$_2$ not extracted; $^{13}$C NMR (100.55 MHz, DMSO, 30 °C): 129.95 (CH), 137.20 (CH), 164.81 (CO), 166.46 (CO).
Products of fumaronitrile transformations by nitrilase NitAg

To obtain samples for LC-MS from the reaction using whole cells carrying NLase NitAg, transformation of FN was carried out analogously as described for NitTv1 above but with modifications (dry cell weight 4.5 g/L, reaction time 120 min, total volume 0.5 ml). The supernatant was diluted with mobile phase (1:50) and analyzed by LC-MS (see Fig. S6 for the chromatogram and figure legend for m/z (ESI) data).

Figure S6. HPLC of the products obtained from fumaronitrile (1a, RT = 2.756) using nitrilase NitAg. Separation conditions: Chromolith RP 18e (100 × 3 mm) column, mobile phase 10% acetonitrile in water, isocratic, flow rate 0.4 mL/min, 34 °C. m/z (ESI): 1b: [M-H]⁻ calculated for C₄H₂NO₂ 96.0, found 96; 1c: [M+Cl]⁻ calculated for C₄H₃N₂OCl 131.1, found 131. See Fig. S3 for the product structures.

Products of fumaronitrile transformations by nitrilase NitSh

Transformation of FN by NitSh was carried out analogously as described for NitTv1 above but with modifications (dry cell weight 3 g/L, reaction time 60 min and total
volume 50 mL). The products were isolated in the same way as in the previous experiment. The product extracted at pH 8 (27 mg) contained a mixture of the residual substrate 1a and product 1c at a ratio of ca. 2 : 3. The product extracted at pH 2.5 contained compound 1b as the major product (isolated yield 69%; 41 mg). The products were analyzed by NMR (Figs S7 and S8).

**Figure S7.** The detail of $^1$H NMR spectrum of the product extracted at pH 8 (399.87 MHz, DMSO, 30 °C)

**Figure S8.** The detail of $^1$H NMR spectrum of the product extracted at pH 2.5 (399.87 MHz, DMSO, 30 °C)
Product of 3-phenylpropionitrile transformation by nitrilase NitTv1

The transformation of 3-phenylpropionitrile (PPN) by NitTv1 was carried out analogously as described for FN above but with modifications (dry cell weight 3 g/L, reaction time 120 min, total volume 0.5 mL). The sample for LC-MS was prepared as described for FN above (see Fig. S9 for the chromatogram and figure legend for m/z (ESI) data).

Figure S9. HPLC of the products obtained from 3-phenylpropionitrile (RT = 9.262 min) using nitrilase NitAg. Separation conditions: Chromolith RP 18e (100 × 3 mm) column, mobile phase 10% acetonitrile in water, isocratic, flow rate 0.4 mL/min, 34 °C.

m/z (ESI): 3-phenylpropionic acid (RT = 5.979 min): [M-H+CH₃OH] calculated. for C₁₀H₁₃O₃ 181.2, found 181.

Products of β-cyanoalanine transformation by nitrilase NitTv1

The transformation of β-cyanoalanine (β-CA) by NLase NitTv1 was carried out analogously as described for FN but with modifications (dry cell weight 0.6 g/L,
reaction time 60 min, total volume 50 mL). The supernatant was lyophilized and analyzed by NMR (Figs S10 and S11).

Figure S10. The detail of $^1$H NMR spectrum of the product extracted at pH 8 (700.13 MHz, D$_2$O, 30 °C). Approximate ratio Asp: Asn = 71: 29. This sample also contained a significant amount of the residual substrate.
**Figure S11.** The detail of $^1$H NMR spectrum of the product extracted at pH 2.5 (700.13 MHz, D$_2$O, 30 °C). Approximate ratio Asp: Asn = 73:27.

**NMR data:** Aspartic acid - $^1$H NMR (700.13 MHz, D$_2$O, 30 °C): 2.705 (1H, dd, $J = 17.5, 8.7$ Hz, H-$\beta_u$), 2.832 (1H, dd, $J = 17.5, 3.8$ Hz, H-$\beta_d$), 3.923 (1H, dd, $J = 8.7, 3.8$ Hz, H-$\alpha$); $^{13}$C NMR (176.05 MHz, D$_2$O, 30 °C): 36.76 (C-$\beta$), 52.46 (C-$\alpha$), 174.43 (CO), 177.79 (CO); Asparagine - $^1$H NMR (700.13 MHz, D$_2$O, 30 °C): 2.891 (1H, dd, $J = 17.0, 7.6$ Hz, H-$\beta_u$), 2.980 (1H, dd, $J = 17.0, 4.3$ Hz, H-$\beta_d$), 4.034 (1H, dd, $J = 7.6, 4.3$ Hz, H-$\alpha$); $^{13}$C NMR (176.05 MHz, D$_2$O, 30 °C): 34.72 (C-$\beta$), 51.52 (C-$\alpha$), 173.49 (CO), 174.66 (CO)

**Products of 2-cyanopyridine transformation by nitrilase NitSh**

The transformation of 2-cyanopyridine (2CP) by NitSh was carried out analogously as described for FN above but with modifications (dry cell weight 0.3 g/L, reaction time 10 min, total volume 0.5 ml). The samples for LC-MS were prepared as described for FN above (see Fig. S12 for the chromatogram and figure legend for $m/z$ (ESI) data).
**Figure S12** HPLC of the products of biotransformation of 2-cyanopyridine by nitrilase NitSh. Separation conditions: Chromolith RP 18e (100 × 3 mm) column, mobile phase 10% acetonitrile in water, isocratic, flow rate 0.4 mL/min, 34 °C. m/z (ESI): picolinic acid (RT= 1.791): [M-H]$^-$ calculated for C$_6$H$_4$NO$_2$ 122.1, found 122; [M-H+H$_2$O]$^-$ calculated for C$_6$H$_6$NO$_3$ 140.0, found 140; picolinamide (RT= 3.252): [M+Na+CH$_3$CN]$^+$ calculated for C$_8$H$_9$N$_3$NaO 186.1, found 186; 2-cyanopyridine, RT= 4.529.

**Products of 4-cyanopyridine transformation by nitrilase NitTv1**

The transformation of 4-cyanopyridine (4CP) by NitTv1 was carried out analogously as described for FN above but with modifications (dry cell weight 3 g/L, reaction time 120 min). The samples for LC-MS were prepared as described for FN above (see Fig. S13 for the chromatogram and figure legend for m/z (ESI) data).

**Figure S13** HPLC of the products of biotransformation of 4-cyanopyridine by nitrilase NitTv1. Separation conditions: Chromolith RP 18e (100 × 3 mm) column, mobile phase 10% acetonitrile in water, isocratic, flow rate 0.4 mL/min, 34 °C. m/z
Products of benzonitrile transformation by nitrilase NitSh

The transformation of benzonitrile (BN) by NitSh was carried out as described for FN above but with modifications (dry cell weight 0.3 g/L, reaction time of 5 min, total volume 0.5 ml). The product (benzoic acid) was determined by HPLC as described in Materials and methods and its UV spectrum was compared with that of the authentic standards (absorption maximum at 228.7). No significant amount of benzamide (absorption maximum at 225.2 nm) was found in the reaction mixture.