Bio-catalytic cascades and molecular oxygen-accessing amines and nitriles

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Chapter 4

Biocatalytic synthesis of nitriles from alcohols utilizing ammonia, air and a promiscuous galactose oxidase variant

This chapter is based on the following publications:

Vilím J., Knaus T., Mutti F. G. (2018). Catalytic Promiscuity of Galactose Oxidase: A Mild Synthesis of Nitriles from Alcohols, Air, and Ammonia. *Angewandte Chemie, International Edition*, 57(43), 14240-14244.

Vilím J., Knaus T., Mutti F. G., UvA Holding BV, Process for the manufacture of nitrile compounds, WO2020020844A1

Vilím J., Wong K. M., Mutti F. G. Recent advances in the synthesis of nitriles. (review) *Manuscript in preparation*.

4.1 Introduction

As was described in Chapter 2, we have noticed the promiscuous formation of benzonitrile from benzyl alcohol in presence of galactose oxidase variant M3-5 originated from *Fusarium* sp. A thorough investigation of the-state-of-the-art literature revealed the potential of this serendipitous discovery.

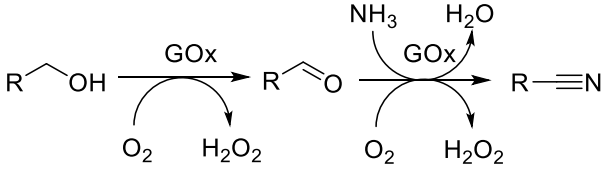
In general, catalytic enzyme promiscuity is defined as the ability of an enzyme to catalyze chemical reactions that are different from the natural one.[1-3] After two decades of intensive investigations, new notable cases of catalytic enzyme promiscuity have been recently revealed and applied in chemical synthesis[4-14] as well as in synthetic biology.[15-17] General methods for the synthesis of nitriles include dehydration of amides,[18,19] formal acid-nitrile exchange,[20-23] Sandmeyer and Rosenmund-von Braun reactions,[24,25] transition-metal catalyzed cyanation,[26,27] electrophilic cyanide transfer[28] and radical-type cleavage reactions.[29] However, these methods generally require toxic cyanide and heat. Cyanide-free routes to nitriles are possible starting from aldehydes (using azide, hydroxylamine or ammonium salts as nitrogen source),[30-35] amines (in presence of metal catalysts or catalytic TEMPO or stoichiometric oxidants),[36-40] azides,[41] pre-formed oximes,[42-46] organic halides,[47,48] or, finally from arenes.[49] Benzonitriles are also produced on industrial scale from toluene by ammoxidation using heterogeneous catalysts, ammonia and dioxygen (450 °C, 2 bar).[50] The direct conversion of alcohols into nitriles attracts interest, but it requires a metal and/or an organic catalyst in the presence of supra-stoichiometric amounts of an organic oxidant and ammonium species.[51-54] However, replacing chemical oxidants with dioxygen would increase significantly the atom-efficiency and the environmental footprint of the reaction. A few systems for the aerobic conversion of alcohols to nitriles have been published, albeit by making use of Cu(II) at high temperature or Fe(III)/TEMPO in MeCN.[55,56]

Due to the raised environmental concerns, biocatalytic approaches constitute important and viable alternatives to the aforementioned chemical methodologies, since they enable the synthesis of nitriles under mild reaction conditions. Those methods include the use of aldoxime dehydratases,[57-60] hydroxynitrile lyases (i.e., addition of cyanide to

carbonyl compounds),[61-64] halohydrin dehalogenases (i.e., ring-opening of epoxides by cyanide),[65,66] and amine oxidases in combination with cyanide salt.[67]
Other enzyme families such as nitrile synthetase,[68] β-cyano-L-alanine synthase[69] and cytochromes [70,71] have limited synthetic applicability. However, there is no report about a one-enzyme conversion of alcohols into nitriles.

4.2 Results and discussion

As mentioned previously, we noticed the unexpected formation of just 1.2% of benzonitrile (**1c**) during the oxidation of benzyl alcohol (**1a**, 10 mM) to benzaldehyde (**1b**) (Scheme 4.1) in ammonium formate buffer (600 mM, pH 9) catalyzed by purified Strep-tagged galactose oxidase (GOx, 25 µM) from *Fusarium sp*. $M_{3\text{-}5}$.[72-77] Naturally, this observation sparked our interest.



Scheme 4.1. Conversion of alcohols to nitriles catalyzed by single galactose oxidase (GOx).

With the aim of increasing benzonitrile formation, we considered that GOx (a Cu-dependent enzyme) requires the addition of exogenous Cu^{2+} to promote the stabilization of its holo-form for biocatalytic reactions *in vitro*.[78-80] The influence of the concentration of added Cu^{2+} towards the activity of GOx $M_{3\text{-}5}$ for the oxidation of alcohols to aldehydes has been determined previously in phosphate buffer.[77] However, the use of phosphate buffer poses the issue of precipitation of nearly insoluble copper phosphate.[81] Thus, we evaluated the influence of the concentration of Cu^{2+} ions (as $CuSO_4$) for the natural oxidation reaction of alcohol **1a** to aldehyde **1b** in Tris-HCl buffer (100 mM, pH 8). Figure 4.1a shows that the conversion of **1a** (10 mM) into **1b**, measured after 40 min, rose progressively at increasing ratio between Cu^{2+} and purified GOx (2.5 µM). The highest yield was observed at a molar ratio of Cu^{2+}/GOx ca. 60:1 (for details, see 4.4.3). Switching from Tris-HCl to $HCOONH_4$ buffer resulted in a similar trend, albeit nitrile **1c** was formed along with **1b**. Therefore, a 50:1 molar ratio of

Cu^{2+}/GOx was used for the continuation of our study. Then, we investigated the influence of the pH towards the formation of **1c** by performing a set of experiments at 30 °C with **1a** (10 mM), GOx (20 μM), Cu^{2+} (1 mM) and catalase (17 μM). The pH was varied from 8 to 10 in $HCOONH_4$ buffer (600 mM). Interestingly, data regarding the catalytic activity of GOx above pH 8 (in any type of buffer) were not available in literature, while the beneficial effect of the addition of catalase was documented.[77] In fact, GOx produces H_2O_2 during the catalytic cycle that may diminish, at certain concentrations, the enzyme activity. Under the reaction conditions reported above, the formation on nitrile versus pH showed a bell-shape with a maximum yield at pH 9 (Figure 4.1b). A second set of experiments aimed at minimizing the amount of catalase for the transformation of **1a** (10 mM) to **1c** at pH 9. Fig. 4.1c shows that the addition of catalase affected positively the reaction, albeit a minimal concentration of 0.83 μM (equal to 0.05 mg ml^{-1}) was sufficient. The evaluation of the influence of the concentration of ammonium species and of temperature on the yield of **1c** shows maxima in the range of 400–600 mM of NH_3/NH_4^+ and at 30 °C (Fig. 4.1d and 4.1e). After the optimization of the reaction parameters, we investigated the influence of air and pure dioxygen (even under pressure) on the progress of the reaction, as dioxygen is the oxidant in the GOx catalytic cycle.[74,77,78,80]. Notably, the supplementation of O_2 as pressurized air or pure O_2 increased slightly the yield of **1c** (Fig. 4.1f). However, a large-scale biocatalytic conversion of alcohols to nitriles operating under pressure would have the disadvantage of consuming energy for pressurization of the system. Therefore, further optimization was conducted using air at atmospheric pressure. The work with highly purified GOx was crucial for demonstrating the promiscuous formation of nitriles from alcohols. Nonetheless, the chemical turnover (TON) for the reaction with purified GOx reached a maximum value of ca. 230 that is insufficient for a synthetic application with oxidoreductases.[82] Hence, we tested GOx as *E. coli* cell free extract (CFE) because costly and time-consuming purification steps are avoided [77] and, possibly, higher GOx activity may be retained. Indeed, optimization of the reaction

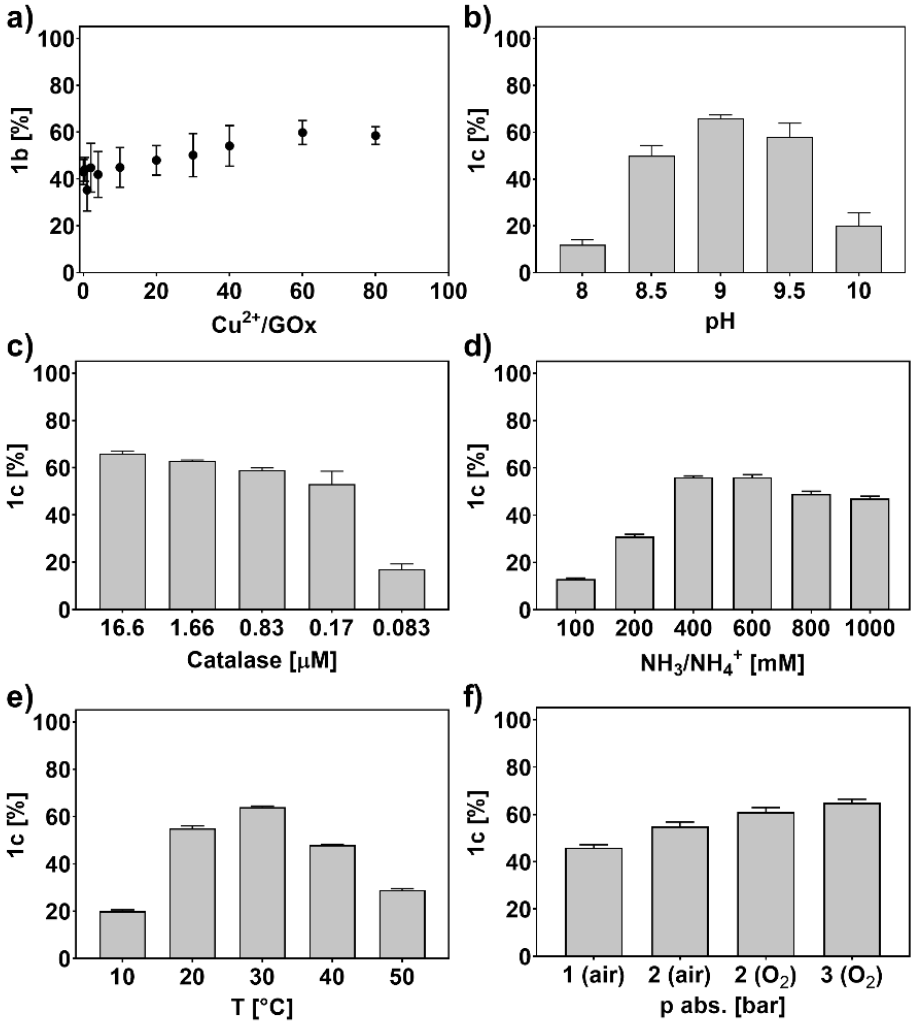


Figure 4.1. Optimization of reaction conditions and determination of substrate scope: a) Influence of copper; b) influence of pH; c) optimal amount of catalase; d) concentration of ammonium species; e) influence of temperature; f) supplementation of molecular oxygen.

conditions for the conversion of **1a** to **1c** using CFE permitted to increase the TON up to ca. 3300 (Fig. 4.2b), which is a value already suitable for a large scale application.[82] Yields of **1c** were in line with the experiments using purified GOx (Fig. 4.2a). In particular, the highest TON of ca. 3300 (1.28 μM GOx as CFE) correlated to 42% yield of **1c**, whereas the highest yield of 65% (2.55 μM GOx as CFE) correlated to a TON of

ca. 2600. Using a 2.55 μM GOx loading as CFE, the promiscuous biocatalytic conversion of alcohols into nitriles was tested with a variety of substrates (10 mM), pre-dissolved in DMSO (2%, v v^{-1}). The reactions were run under the optimized conditions (NH_3/NH_4^+ 400 mM, pH 9, catalase 0.83 μM, 30 °C). With the exception of cyclohexylmethanol (**16a**), 2-pyridylmethanol (**19a**), 2-phenylethanol (**20a**) and 3-phenyl-1-propanol (**21a**), all the other alcohols were converted into nitriles (for yields, TONs: Fig. 4.2c).

Interestingly, within a homologous series, benzyl alcohols containing electron-withdrawing substituents in *ortho* position were converted with higher yields (**4c**, **7c**, **10c**) compared to the *para*- (**2c**, **5c**, **8c**) and especially the *meta*-substituted ones (**3c**, **6c**, **9c**). The effect was reversed with the electron-donating methyl substituent, as *ortho*-methyl benzyl alcohol was less converted (**13c**) than *para*-methyl (**11c**) and *meta*-methyl benzyl alcohol (**12c**). The highest yield was 70% for the conversion of 2-fluorobenzyl alcohol (**4a**) into 2-fluorobenzyl nitrile (**4c**). Cinnamyl alcohol (**22a**) was also accepted, leading to 10% yield into the related nitrile **22c**. Moreover, 4-pyridyl methanol and 3-pyridyl methanol were also transformed into corresponding nitriles (**17c**, **18c**) with 10% and 55% yield, respectively. Besides the formation of the nitrile products, variable amounts of carboxylic acids (**1-14e**, **17-18e**, **22e**) were detected in agreement with the findings reported in a concomitant publication focused on oxidation of alcohols to carboxylic acids catalyzed by GOx.[83] We point out that nitriles and carboxylic acids can be separated easily by extracting the former directly from the reaction buffer (pH 9) and, in case, the latter after acidification. In many cases, both nitriles and carboxylic acids are valuable compounds (e.g., oxidation of **18a** to vitamins B3: **18c** and **18e**). However, interestingly, the yield of nitriles (and the chemo-selectivity of the reaction) were somehow dependent on the scale of the reaction. For instance, a preparative scale synthesis was performed with **4a** (151 mg, 1.2 mmol) under the optimized reaction conditions using CFE. After 24 h, the reaction afforded >99% analytical yield of nitrile **4c** (exactly quantified with an internal standard). After extraction

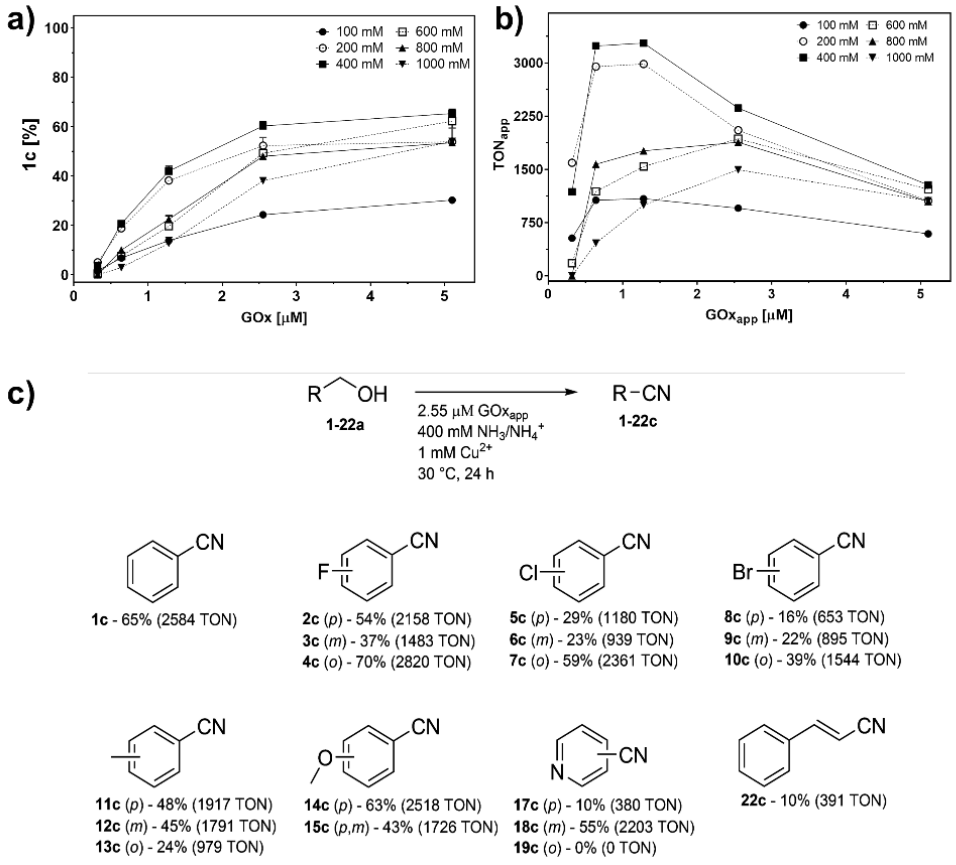


Figure 4.2. Synthesis of nitriles from alcohols using CFE. Analytical yield of **1c** (a) and TON (b) at varied concentration of NH_3/NH_4^+ and GOx. Substrate scope (c). For experimental details, see section 4.4.

and solvent evaporation, nitrile **4c** was isolated in 75% yield and pure form (no further purification step was required). Conversely, the biocatalytic conversion of **4a** on analytical scale (Fig. 2C) produced 70% analytical yield of nitrile **4c** and 5% of carboxylic acid **4e**. We attribute the discrepancy to different aeration and agitation between analytical scale and preparative scale reactions.

Regarding the mechanism for the formation of nitrile from alcohol, we further proved the promiscuous activity of GOx by exploring a possible non-enzymatic or non-specific conversion of the aldehyde **1b** into nitrile **1c**. In fact, there are literature reports

describing that H_2O_2, Cu^{2+} or formate may contribute to the conversion of **1b** to **1c** (and derivatives thereof), but in presence of additional reagents and under particular reaction conditions.[55,84] A series of reactions (Table 4.1) revealed that nitrile **1c** is indeed produced from aldehyde **1b** only in presence of GOx (entry 1, 47% yield). Partial loss of GOx activity was observed when H_2O_2 was also added into the mixture (entry 2, 33% yield), confirming the detrimental effect of H_2O_2 in high concentration.[77] Several control experiments including albumin and/or Cu^{2+} and/or H_2O_2 afforded just traces of **1c** (<0.5%) only in presence of H_2O_2 (entries 4 and 6).

Table 4.1. Study on the formation of nitrile from **1b** or **1d** using GOx or albumine. If not stated otherwise, Cu^{2+} (50 eq.) was added. For details, see, SI. [a]: H_2O_2 (10 mM) was added. [b]: Cu^{2+} was omitted.

Entry	Substrate	pH	GOx (μM)	Albumine (μM)	Analytical Yield (%)
1	1b	9	20	-	47
2	1b[a]	9	20	-	33
3	1b	9	-	-	0
4	1b[a]	9	-	-	<0.5
5	1b	9	-	20	0
6	1b[a]	9	-	20	<0.5
7	1d	7	20	-	0
8	1d	9	20	-	0
9	1d[b]	7	20	-	0
10	1d[b]	9	20	-	0

In the same way, formation of **1c** from **1a** was observed only in the presence of GOx. Finally, the transformation of an aldehyde into a nitrile may occur via two possible paths: 1) imine formation by reaction with ammonia and subsequent promiscuous oxidation to the nitrile via hydride abstraction, or 2) imine formation, subsequent promiscuous hydroxylation to an oxime and final dehydration to give the nitrile. Thus, benzaldehyde oxime (**1d**) was incubated with GOx under various conditions (Table 1, entries 7-10; for details see section 4.4.12), but dehydration to the nitrile was never observed. Consequently, nitrile **1c** is formed by direct oxidation of the imine intermediate.

4.3 Conclusion

In conclusion, we have discovered a new promiscuous activity of the galactose oxidase that is the one-pot synthesis of benzyl, pyridyl and cinnamyl nitriles from the related alcohols using only ammonia as source of nitrogen and dioxygen as innocuous oxidant. Compared with recently reported approaches used to transform alcohols or aldehydes into nitriles,[26,30,34,55,56] the GOx-catalyzed reaction has significant advantages such as mild reaction conditions in aqueous medium, simple operational set-up and elevated atom-economy. Moreover, utilization of GOx in form of CFE increased the TON to synthetically applicable levels and avoided any purification steps. This promiscuous activity of GOx has already notable applications as cinnamonitrile is an important synthetic aroma,[85] whereas benzonitriles constitute the active core of the large majority of nitrile-containing pharmaceuticals.[86,87] Moreover, 3-cyanopyridine is a precursor to vitamin B3, to which it can be converted by established enzymatic methods.[88,89] Future research will focus on searching for other promiscuous copper-dependent alcohol oxidases, which are active on structurally different alcohols, in order to enable even broader application of this new biocatalytic reaction.

4.4 Experimental

4.4.1 Cloning and expression

The gene encoding for GOx[72,77] connected to a C-terminal triple Strep-tag was cloned into pET42a. For recombinant expression of GOx, LB medium (800 ml) with kanamycin (50 mg l^{-1}) was inoculated with an overnight culture of *E. coli* BL21 DE3 cells (15 ml) harboring the expression plasmid, and grown at 37 °C until OD_{600} reached a value of 0.6–0.9. At that point, the media was supplemented with $CuSO_4 \cdot 5H_2O$ (cofactor for GOx, 250 mg l^{-1} final concentration) and, subsequently, expression was induced by the addition of IPTG (0.5 mM final concentration). Expression was carried out overnight at 25 °C and after harvesting of the cells (4 °C, 3400 g, 15 min), the cell pellet was washed with NaCl solution (0.9%), centrifuged at the same conditions and frozen at -20°C. The expression was verified by the SDS-PAGE (Figure 4.3A). Obtained cells were used for the purification of the protein with affinity chromatography or for the preparation of cell free extract (CFE).

4.4.2 Purification of recombinant GOx

A modification of a previous purification protocol was used.[72] Frozen cell pellets containing GOx were thawed, re-suspended in binding buffer (100 mM Tris-HCl, 150 mM NaCl, pH 8) and the cells dispersion was sonicated for 15 minutes (10 s on, 15 s off, 45% amp) until reduction of viscosity was observed. Cell debris was removed by centrifugation (ca. 35000 g, 4°C). The supernatant was filtered through a 0.45 µm filter (Whatman) and the enzyme was purified using a StrepTrapHP column (GE Healthcare) according to the purification manual. Purified protein fractions were pooled and dialyzed overnight at 4°C against the binding buffer containing six-fold molar excess of Cu^{+2} ions compared with the enzyme, to ensure the loading of the active site of the enzyme with Cu^{2+}. The next day, the dialysis was repeated against the binding buffer to remove excess of copper. After 24 hours, the enzyme was concentrated using Vivaspin (Milipore) columns, the concentration was determined using the Biorad protein assay kit (BioRad) and the enzyme was dropwise flash frozen in liquid nitrogen. Purity of the enzyme was assessed by SDS-PAGE (Figure 4.3B). GOx was purified with yield of 8.6

mg g^{-1} of wet cells. The yield was used to calculate the actual concentration of GOx in the CFE (CFE was used in the experiments described at sections 4.4.10, 4.4.11 and 4.4.15).

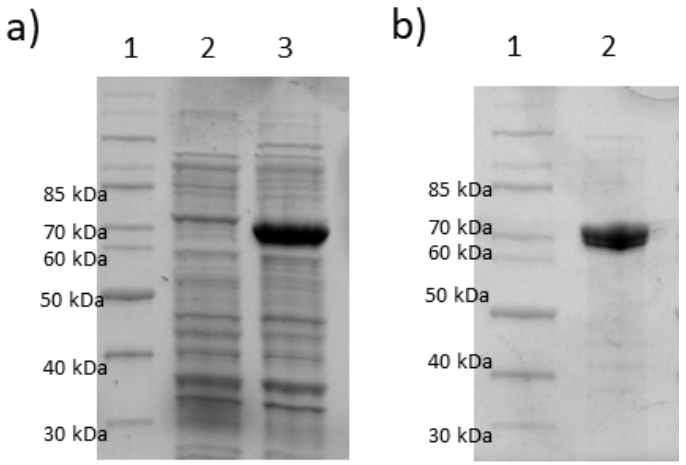


Figure 4.3. (a) SDS-PAGE gel of pre- and after induction samples: lane 1: PageRuler™ Unstained Protein Ladder (ThermoFisher Scientific), lane 2: before induction with IPTG (0.5 mM); lane 3: after 24 h induction with IPTG (0.5 mM) for 24 h. (b) lane 1: PageRuler™ Unstained Protein Ladder; lane 2: Purified GOx, 5 µg of protein loaded onto gel.

4.4.3 Influence of supplementation of Cu^{2+} on the activity of GOx

Reaction conditions: 500 µL final volume in 1.5 mL Eppendorf tubes; Tris-HCl buffer (100 mM, pH 8), GOx (2.5 µM final concentration), varied amount of $CuSO_4{\cdot}5H_2O$ (0–80:1 molar ratio calculated to GOx concentration), **1a** (10 mM). The biotransformations were incubated at 170 rpm and 30 °C in an orbital shaker for 40 min, stopped by the addition of KOH (10 M, 70 µl) and extracted twice with EtOAc (300 µl and 350 µl, respectively). The combined organic phases were dried over $MgSO_4$ and analytical yields were determined by GC-FID (see section 4.4.15) using calibration curves. Reactions were performed in two sets of duplicates for each Cu^{2+}/GOx ratio and the results are summarized in Table 4.2.

Table 4.2. Influence of the concentration of Cu^{2+} on the activity of GOx. Cu^{2+}/GOx ratio represents the molar ratio between Cu^{2+} ions and GOx. Reactions were run in Tris-HCl buffer (100 mM, pH 8), GOx (2.5 μM), **1a** (10 mM), T = 30°C, t = 40 min.

Cu^{2+}/GOx ratio	Analytical Yield (%)
0	43 ± 5
0.4	44 ± 5
1	35 ± 9
2	45 ± 10
4	42 ± 10
10	45 ± 9
20	48 ± 6
30	50 ± 9
40	54 ± 9
60	60 ± 5
80	59 ± 4

4.4.4 Determination of the optimal pH value for the formation of nitriles

Reaction conditions: 500 μL final volume in 1.5 mL Eppendorf tubes; ammonium formate buffer (600 mM) at different pH values ranging from 8–10 (the pH value was adjusted by the addition of either KOH or formic acid), GOx (20 μM final concentration, buffer exchange was performed with Vivaspin centrifugation columns by washing the protein with 5 volumes of reaction buffer), Cu^{2+} (50:1 molar ratio calculated to GOx concentration), catalase (16.6 μM) and **1a** (10 mM). The biotransformations were incubated at 170 rpm and 30 °C in an orbital shaker for 24 h, stopped by the addition of KOH (10 M, 70 μl) and extracted twice with EtOAc (300 μl and 350 μl, respectively) containing 10 mM toluene. The combined organic phases were dried over $MgSO_4$ and analytical yields were determined by GC-FID (see section 4.4.15) using toluene as internal standard. Reactions were performed in triplicate and the results are summarized in Table 4.3.

Table 4.3. Influence of the pH value on the formation of **1c** in ammonium formate buffer (600 mM, pH 8–10) with GOx (20 µM), Cu^{2+} (1 mM), **1a** (10 mM), catalase (16.6 µM), T = 30°C, t = 24 h.

pH	Analytical Yield (%)
8	12 ± 2
8.5	50 ± 4
9	66 ± 1
9.5	58 ± 6
10	20 ± 6

4.4.5 Determination of the optimum amount of catalase

Reaction conditions: 500 µL final volume in 1.5 mL Eppendorf tubes; ammonium formate buffer (600 mM, pH 9), GOx (20 µM final concentration), Cu^{2+} (50:1 molar ratio calculated to GOx concentration), varied concentrations of catalase (0.085–17 µM) and **1a** (10 mM). The biotransformations were incubated at 170 rpm and 30 °C in an orbital shaker for 24 h, stopped by the addition of KOH (10 M, 70 µl) and extracted twice with EtOAc (300 µl and 350 µl, respectively) containing 10 mM toluene. The combined organic phases were dried over $MgSO_4$ and analytical yields were determined by GC-FID (see section 4.4.15) using toluene as internal standard. Reactions were performed in triplicate and the results are summarized in Table 4.4.

Table 4.4. Influence of amount of catalase on the formation of **1c** in ammonium formate buffer (600 mM, pH 9) with GOx (20 µM), Cu^{2+} (1 mM), **1a** (10 mM), varied concentration of catalase (0.083–16.6 µM), T = 30°C, t = 24 h.

Catalase (µM)	Analytical Yield (%)
0.083	17 ± 2
0.17	53 ± 6
0.83	59 ± 1
1.66	63 ± 0.3
16.6	66 ± 1

4.4.6 Determination of the optimum concentration of ammonium species

Reaction conditions: 500 µL final volume in 1.5 mL Eppendorf tubes; ammonium formate buffer (pH 9) at varied concentration (100 mM–1 M), GOx (20 µM final concentration), Cu^{2+} (50:1 molar ratio calculated to GOx concentration), catalase (0.83 µM) and **1a** (10 mM). The biotransformations were incubated at 170 rpm and 30 °C in an orbital shaker for 24 h, stopped by the addition of KOH (10 M, 70 µl) and extracted twice with EtOAc (300 µl and 350 µl, respectively) containing 10 mM toluene. The combined organic phases were dried over $MgSO_4$ and analytical yields were determined by GC-FID (see section 4.4.15) using toluene as internal standard. Reactions were performed in triplicate and the results are summarized in Table 4.5.

Table 4.5. Influence of the concentration of ammonium/ammonia species on the formation of **1c** in ammonium formate buffer (pH 9, varied concentration 100 mM–1 M) with GOx (20 µM), Cu^{2+} (1 mM), **1a** (10 mM), catalase (0.83 µM), T = 30°C, t = 24 h.

Ammonium species (mM)	Analytical Yield (%)
100	13 ± 0.4
200	31 ± 1
400	56 ± 1
600	56 ± 1
800	49 ± 1
1000	47 ± 1

4.4.7 Influence of temperature on the formation of nitriles

Reaction conditions: 500 µL final volume in 1.5 mL Eppendorf tubes; ammonium formate buffer (600 mM, pH 9), GOx (20 µM final concentration), Cu^{2+} (50:1 molar ratio calculated to GOx concentration), catalase (0.83 µM) and **1a** (10 mM). The biotransformations were incubated at 170 rpm and at different temperatures (10–50 °C) in an orbital shaker for 24 h, stopped by the addition of KOH (10 M, 70 µl) and extracted twice with EtOAc (300 µl and 350 µl, respectively) containing 10 mM toluene. The combined organic phases were dried over $MgSO_4$ and analytical yields were determined by GC-FID (see section 4.4.15) using toluene as internal standard. Reactions were performed in triplicate and the results are summarized in Table 4.6.

Table 4.6. Influence of temperature on the formation of **1c** in ammonium formate buffer (pH 9, 200 mM) with GOx (20 µM), Cu^{2+} (1 mM), **1a** (10 mM), catalase (0.83 µM), at varied temperature (20–50 °C), t = 24 h.

Temperature (°C)	Analytical Yield (%)
10	20 ± 1
20	55 ± 1
30	65 ± 0.4
40	48 ± 0.2
50	29 ± 0.5

4.4.8 Influence of O_2 supplementation

To investigate whether an elevated dioxygen level can increase the activity of the enzyme, experiments were additionally performed in a chamber pressurized with (a) atmospheric pressure (b) 2 bar of air, (c) 2 bar of pure O_2 or (d) 3 bar of pure O_2 (conditioned with 1 min of flushing).

Reaction conditions: 500 µL final volume in 2 ml Rotilabo sample vials, closed with screw caps containing a PTFE septa (Carl Roth GmbH). A needle (22G1, Becton Dickson) served as valve to allow dioxygen to access into the vial during the reaction. The reaction consisted of ammonium formate buffer (600 mM, pH 9), GOx (20 µM final concentration), Cu^{2+} (50:1 molar ratio calculated to GOx concentration), catalase (0.83 µM) and **1a** (10 mM). The vials were transferred into a chamber and pressurized as described above. The biotransformations were incubated at 170 rpm and 30 °C in an orbital shaker for 24 h. Then, the reactions were transferred into 1.5 ml Eppendorf tubes, stopped by the addition of KOH (10 M, 70 µl) and extracted twice with EtOAc (300 µl and 350 µl, respectively) containing 10 mM toluene. The combined organic phases were dried over $MgSO_4$ and analytical yields were determined by GC-FID (see section 4.4.15) using toluene as internal standard. Reactions were performed in triplicate and the results are summarized in Table 4.7.

Table 4.7. Influence of O_2 supplementation on the formation of **1c** in ammonium formate buffer (200 mM, pH 9) with GOx (10 μM), Cu^{2+} (0.5 mM), **1a** (10 mM), catalase (0.83 μM), T = 30°C, t = 24 h, 1-2 bar of air or 2-3 bar of O_2.

Absolute O_2 pressure (bar)	Analytical Yield (%)
1 (air)	46 ± 1
2 (air)	55 ± 2
2 (O_2)	61 ± 2
3 (O_2)	65 ± 1

4.4.9 Substrate scope using purified enzyme

Reaction conditions: 500 μL final volume in 1.5 mL Eppendorf tubes; ammonium formate buffer (600 mM, pH 9), GOx (20 μM final concentration), Cu^{2+} (50:1 molar ratio calculated to GOx concentration), catalase (0.83 μM) and **1-22a** (10 mM). The biotransformations were incubated at 170 rpm and at different temperatures (30 °C) in an orbital shaker for 24 h, stopped by the addition of KOH (10 M, 70 μl) and extracted twice with EtOAc (300 μl and 350 μl, respectively) containing 10 mM toluene. The combined organic phases were dried over $MgSO_4$ and analytical yields were determined by GC-FID (see section 4.4.15) using toluene as internal standard. Reactions were performed in triplicate and the results are summarized in Figure 4.4. Only observed products are showed—nitriles **16c**, **19c**, **20c** and **21c** were not observed.

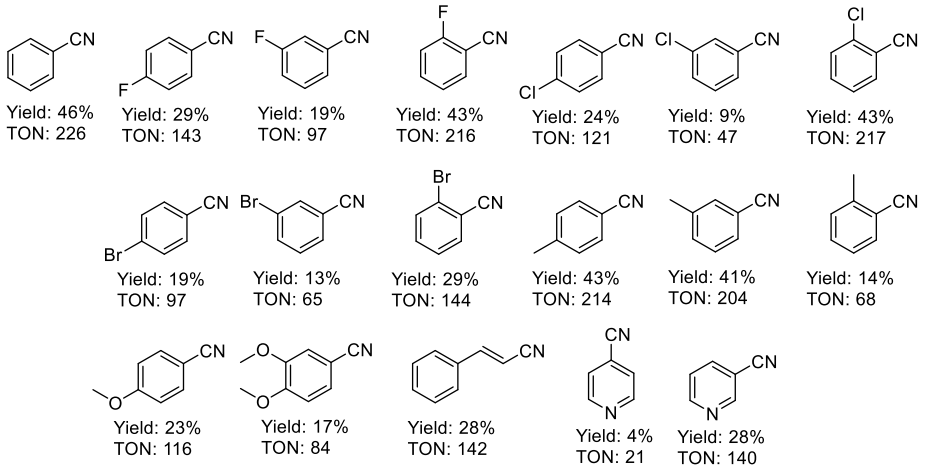


Figure 4.4. Preliminary experiments on substrate scope using purified GOx (20 μM), Cu^{2+} (50:1 molar ratio calculated to GOx concentration), catalase (0.83 μM) and **1-22a** (10 mM; added as stock solutions in DMSO with 2% v/v final concentration) in ammonium formate buffer (pH 9). Analytical yield (determined using toluene as internal standard) and calculated chemical turnover (TON) are reported.

4.4.10 Cell free extract: optimization of performance

GOx crude cell free extraxt (CFE) was prepared by suspending the *E. coli* cells (700 mg) containing the overexpressed GOx in the ammonium formate buffer (pH 9, varied concentration as reported in Table 4,8). Then, cells were sonicated (10 min, 10 s on, 10 s off, 45% amp) and centrifuged (ca. 35000 g, 15 min, 4°C). The CFE (supernatant) was collected and used for performing the biocatalytic reactions.

Reaction conditions: for a reaction in analytical scale (in 1.5 ml Eppendorf tubes), 0.02–0.32 ml of CFE were used (ca. equivalent to a final GOx concentration of 0.32–5.12 μM calculated based on purification yield of 8.6 mg_{enzyme} $g^{-1}{}_{wet\ cells}$ and MW $Strep_3$-tagged GOx of 73.6 kDa). Reaction mixture also contained $CuSO_4 \cdot 5H_2O$ (1 mM), catalase (0.83 μM) and substrate **1a** (10 mM, added as DMSO stock solution with a final DMSO content of 2% v v^{-1}) in a final reaction volume of 0.5 mL ($HCOONH_4$, pH 9, varied concentration). Samples were incubated at 170 rpm, 20 °C for 24 h, stopped by the addition of KOH (10 M, 70 μl) and extracted twice with EtOAc containing 10 mM toluene as internal standard (300 μl and 350 μl, respectively). The combined organic phases

were dried over $MgSO_4$ and analytical yields were determined via GC-FID (see section 4.4.15). Experiments were performed in duplicates and the results are summarized in Table 4.8 and 4.9.

Table 4.8. Influence of CFE loading and buffer concentration on conversion of **1a** to **1c** in ammonium formate buffer (100 mM–1M, pH 9) with CFE (20–320 µL, equal to 0.32–5.12 µM GOx), Cu^{2+} (1 mM), **1a** (10 mM), catalase (0.83 µM), T = 30°C, t = 24 h.

CFE (µL)	GOx (µM)	Analytical Yield (%)					
		Ammonium species (mM)					
		100	200	400	600	800	1000
20	0.32	2	5	4	1	0	0
40	0.64	7	19	21	8	10	3
80	1.28	14	38	42	20	23	13
160	2.55	24	52	60	49	48	38
320	5.10	30	54	65	62	54	54

Table 4.9. Influence of CFE loading and buffer concentration on the chemical turnover number (TON) in ammonium formate buffer (100 mM–1M, pH 9) with CFE (20–320 µL, equal to 0.32–5.12 µM GOx), Cu^{2+} (1 mM), **1a** (10 mM), catalase (0.83 µM), T = 30°C, t = 24 h.

CFE (µL)	GOx_a (µM)	TON					
		Ammonium species (mM)					
		100	200	400	600	800	1000
20	0.32	533	1594	1185	179	0	0
40	0.64	1068	2949	3240	1189	1572	462
80	1.28	1087	2986	3281	1540	1762	997
160	2.55	955	2053	2365	1933	1883	1495
320	5.10	593	1057	1281	1220	1049	1059

4.4.11 Substrate scope using cell free extract

GOx crude cell free extract (CFE) was prepared as reported in the previous section. Reactions were run as reported in the previous section at 1 mL scale using 320 µl of CFE (ca. equivalent to a final GOx concentration of 2.56 µM GOx) in ammonium formate buffer (pH 9, 400 mM). Samples were incubated at 170 rpm, 20 °C for 24 h. For the analysis of conversion to nitriles for reactions with substrates **1-16a** and **20-22a**, a 0.5 ml aliquot of the reaction mixture was taken from the reaction tube and

stopped by the addition of KOH (10 M, 70 µl) and extracted twice with EtOAc containing 10 mM toluene as internal standard (300 µl and 350 µl, respectively). In the case of the reaction with substrates **17a**-**19a**, addition of KOH before extraction was omitted to prevent partial hydrolysis of products **17-19c** to amides. The combined organic phases were dried over $MgSO_4$ and analytical yields of nitriles **1-22c** were determined via GC-FID (see paragraph Analytics). For the quantification of presence of carboxylic acids, a 0.4 ml aliquot of the reaction mixture was taken from the reaction tube and it was acidified with HCl (3 N, 100 µl) and extracted once with 520 µl of EtOAc containing 10 mM toluene. Extract was dried over $MgSO_4$ and aliquot of extract was derivatized with TMSDM and analytical yields were determined with GC-FID (see section 4.4.15). Experiments were performed in duplicates and the results are summarized in Table 4.10.

Table 4.10. Substrate scope using cell free extract. Analytical yield of substrate to the corresponding nitrile (**1-22c**) and carboxylic acid (**1-22e**) was calculated using 10 mM toluene as internal standard. TON indicates the chemical turnover for nitrile (**1-22c**) formation. n.d., not determined; n.a.; not applicable, substrate was not accepted.

Entry	Substrate	Analytical Yield to nitrile **1-22c** (%)	TON for nitrile formation	Analytical Yield to carboxylic acid **1-22e** (%)
1	Benzylalcohol	65	2584	14
2	4-Fluorobenzyl alcohol	54	2158	22
3	3-Fluorobenzyl alcohol	37	1483	55
4	2-Fluorobenzyl alcohol	70	2820	5
5	4-Chlorobenzyl alcohol	29	1180	48
6	3-Chlorobenzyl alcohol	23	939	69
7	2-Chlorobenzyl alcohol	59	2361	1
8	4-Bromobenzyl alcohol	16	653	48
9	3-Bromobenzyl alcohol	22	895	78
10	2-Bromobenzyl alcohol	39	1544	1
11	4-Methylbenzyl alcohol	48	1917	7
12	3-Methylbenzyl alcohol	45	1791	15
13	2-Methylbenzyl alcohol	24	979	5
14	4-Methoxybenzyl alcohol	63	2518	6
15	3,4-Dimethoxybenzyl alcohol	43	1726	n.d.
16	Cyclohexylmethanol	0	0	n.a.

Entry	Substrate	Analytical Yield to nitrile **1-22c** (%)	TON for nitrile formation	Analytical Yield to carboxylic acid **1-22e** (%)
17	4-Pyridinemethanol	10	380	90
18	3-Pyridinemethanol	55	2203	45
19	2-Pyridinemethanol	0	0	0
20	2-Phenylethanol	0	0	n.a.
21	3-Phenyl-1-propanol	0	0	n.a.
22	*trans*-Cinnamic alcohol	10	391	2

4.4.12 Validation experiments for the catalytic enzymatic promiscuous activity

Formation of **1c** from aldehyde intermediate **1b**. Reaction conditions: 500 µL final volume in 1.5 mL Eppendorf tubes; ammonium formate buffer (600 mM, pH 9), purified GOx (0 or 20 µM final concentration) or commercially available purified bovine albumin (0 or 20 µM final concentration), $CuSO_4 \cdot 5H_2O$ (0 or 1 mM final concentration), H_2O_2 (0 or 10 mM) and **1b** (10 mM). The samples were incubated at 170 rpm and 30 °C in an orbital shaker for 24 h, stopped by the addition of KOH (10 M, 70 µl) and extracted twice with EtOAc (300 µl and 350 µl, respectively) containing 10 mM toluene as internal standard. The combined organic phases were dried over $MgSO_4$ and analytical yields were determined by GC-FID (see section 4.4.15). Reactions were performed in duplicates and the results are summarized in Table 4.11.

Table 4.11. Validation experiments for the biocatalytic promiscuous activity of GOx for the formation of **1c** from **1b**. Ammonium formate buffer (600 mM, pH 9), GOx (0 or 20 µM) or bovine albumin (0 or 20 µM), Cu^{2+} (0 or 1 mM), H_2O_2 (0 or 10 mM), **1b** (10 mM), 30 °C, 24 h.

Entry	GOx (20 µM)	Bovine albumin (20 µM)	Cu^{2+} (1 mM)	H_2O_2 (10 mM)	Analytical yield (%)
1	+	-	+	-	47 ± 0.1
2	+	-	+	+	33 ± 1
3	-	-	+	+	< 0.1
4	-	-	-	+	< 0.2
5	-	+	+	-	0
6	-	+	+	+	< 0.4

4.4.13 Testing aldoxime as possible intermediate for the conversion of alcohol into nitrile

Reaction conditions: 500 µL final volume in 1.5 mL Eppendorf tubes; Tris-HCl buffer (45 mM, pH 7 or 9), purified GOx (0 or 20 µM final concentration), $CuSO_4 \bullet 5H_2O$ (0 or 1 mM final concentration), catalase (0 or 0.83 µM) and benzaldehyde oxime (**1d**, 10 mM). The samples were incubated at 170 rpm and 30 °C in an orbital shaker for 24 h, stopped by the addition of KOH (10 M, 70 µl) and extracted twice with EtOAc (300 µl and 350 µl, respectively). The combined organic phases were dried over $MgSO_4$ and the presence of aldoxime was verified by GC (see section 4.4.15). Reactions were performed in duplicates and the results are summarized in Table 4.12.

Table 4.12. Formation of **1c** from **1d** by GOx in Tris-HCl buffer (45 mM, pH 7 or 9): GOx (0 or 20 µM), Cu^{2+} (0 or 1 mM), catalase (0.83 µM), **1d** (10 mM), 30°C, 24 h. n.d., not detected.

Entry	GOx (20 µM)	Cu^{2+} (1 mM)	Catalase (0.83 µM)	pH	Activity
1	+	+	-	7	n.d.
2	+	+	-	9	n.d.
3	+	-	-	7	n.d.
4	+	-	-	9	n.d.
5	-	+	-	7	n.d.
6	-	+	-	9	n.d.
7	-	-	-	7	n.d.
8	-	-	-	9	n.d.
9	-	-	+	7	n.d.
10	-	-	+	9	n.d.

4.4.14 Preparative scale synthesis of 4c

5.1 g of *E. coli* cells harboring GOx were resuspended in 51 ml of 400 mM ammonium formate buffer pH 9 and lysed by sonication (10 min, 10 s on, 10 s off, 45% amp). After centrifugation (approx. 35000 g, 40 min, 4 °C), the supernatant was used (CFE) for the biocatalytic reaction.

The reaction was performed in ammonium formate buffer (400 mM, pH 9) and consisted of: total volume 120 mL in 500 mL Erlenmeyer flask, CFE (48 ml, or 40% of

reaction volume), $CuSO_4 \cdot 5H_2O$ (1 mM), catalase (0.83 µM) and substrate **4c** (151 mg, 1.2 mmol). The biocatalytic reaction was incubated at 30 °C, 170 rpm for 22 h. After 22 h, an analytical yield to **4c** of >99% was measured. After 23 h, the organic components were extracted with diethylether (3x, total volume 360 ml). The solvent was removed under reduced pressure resulting in 108.8 mg **1c** (75% yield).

4.4.15 Analytics

4.4.15.1 Determination of analytical yield by GC-FID

GC-FID was performed on an Agilent 7890B chromatograph using H_2 as carrier gas.
Columns: Agilent J&W DB1701 (30 m, 250 µm, 0.25 µm); Agilent J&W HP-5 (30 m, 320 µm, 0.25 µm).

Method A: T injector 250 °C; constant pressure 6.9 psi; temperature program: 80 °C, hold 6.5 min; 10 °C min^{-1} to 160 °C, hold 5 min; 20 °C min^{-1} to 200 °C, hold 2 min; 20 °C min^{-1} to 280 °C, hold 1 min.
Method B: T injector 250 °C; constant pressure 6.9 psi; temperature program: 60 °C, hold 6.5 min; 20 °C min^{-1} to 100 °C, hold 1 min; 20 °C min^{-1} to 280 °C, hold 1 min.
Method C: injector 250 °C; constant pressure 4.0 psi; temperature program: 60 °C, hold 0 min; 10 °C min^{-1} to 300 °C, hold 1 min.
Method D: T injector 250 °C; constant flow 1.3 ml min^{-1}; temperature program: 60 °C, hold 0 min; 10 °C min^{-1} to 120 °C, hold 30 min; 10 °C min^{-1} to 300 °C, hold 1 min.
Retention time - IS (toluene) – 3.0 min (methods A, C); 3.2 min (method D); 4.0 min (method B).
- co-solvent (DMSO) – 3.4 min (method C); 3.6 min (method D); 7.3 min (method A); 9.6 min (method B).
- retention times of substrates, products and intermediates are listed in Table 13.

Table 4.13. List of retention times, type of column and acquisition method used for GC analysis.

Entry	Substrate	Column	Method	Split ratio	retention time (min)		
					Alcohol (**a**)	Aldehyde (**b**)	Nitrile (**c**)
1	Benzylalcohol	DB1701-30m	Method A	20	11.5	8.5	9.8
2	4-Fluorobenzyl alcohol	DB1701-30m	Method A	20	12.2	8.2	9.6
3	3-Fluorobenzyl alcohol	DB1701-30m	Method A	20	12.5	8.0	8.7
4	2-Fluorobenzyl alcohol	DB1701-30m	Method A	20	11.7	7.7	10.5
5	4-Chlorobenzyl alcohol	DB1701-30m	Method A	20	16.1	12.7	13.5
6	3-Chlorobenzyl alcohol	DB1701-30m	Method A	20	16.2	12.6	13.2
7	2-Chlorobenzyl alcohol	DB1701-30m	Method A	20	15.3	12.3	14.4
8	4-Bromobenzyl alcohol	DB1701-30m	Method A	20	18.5	14.6	15.4
9	3-Bromobenzyl alcohol	DB1701-30m	Method A	20	18.7	14.6	15.1
10	2-Bromobenzyl alcohol	DB1701-30m	Method A	20	17.3	14.2	16.4
11	4-Methylbenzyl alcohol	DB1701-30m	Method A	20	13.4	11.6	12.7
12	3-Methylbenzyl alcohol	DB1701-30m	Method A	20	13.4	11.3	12.3
13	2-Methylbenzyl alcohol	DB1701-30m	Method A	20	13.7	11.1	11.6
14	4-Methoxybenzyl alcohol	HP-5	Method C	20	9.6	9.2	9.5
15	3,4-Dimethoxybenzyl alcohol	HP-5	Method D	30	23.2	21.2	22.7
16	Cyclohexylmethanol	DB1701-30m	Method B	20	11.2	9.5	11.7
17	4-Pyridinemethanol	DB1701-30m	Method A	20	15.3	9.5	9.7
18	3-Pyridinemethanol	DB1701-30m	Method A	20	14.8	10.3	10.9
19	3-Pyridinemethanol	DB1701-30m	Method A	20	11.6	8.2	13.0
20	2-Phenylethanol	DB1701-30m	Method A	20	13.0	11.0	16.2
21	3-Phenyl-1-propanol	DB1701-30m	Method A	20	15.2	13.6	14.0
22	*trans*-Cinnamic alcohol	HP-5	Method C	20	9.5	9.0	9.4

4.4.15.2 Quantification of carboxylic acids

GC-FID

Derivatization: 400 µl aliquot of acidic extract was pipetted into 2.0 ml Eppendorf tube and 200 µl of EtOAc and 200 µl of MeOH were added. The derivatization was started by the addition of 30 µl TMSDM and the reactions were shaken at 30 °C, 170 rpm for 1.5 h. The excess of derivatizing agent was then destroyed by the addition of 4 µl of anhydrous acetic acid and samples were subjected to GC-FID analysis according to method listed below. The retention times are listed in Table 14.

GC-FID was performed on an Agilent 7890B chromatograph using H_2 as carrier gas using the same set of methods as described in section 6.2.

Columns: Agilent J&W DB1701 (30 m, 250 µm, 0.25 µm); Agilent J&W HP-5 (30 m, 320 µm, 0.25 µm)

Table 4.14. List of retention times, type of column and acquisition method used for GC analysis of carboxylic acids

Entry	Substrate	Column	Method	Split ratio	Derivatized **1-22e** (min)
1	Benzylalcohol	DB1701-30m	Method A	20	12.0
2	4-Fluorobenzyl alcohol	DB1701-30m	Method A	20	11.4
3	3-Fluorobenzyl alcohol	DB1701-30m	Method A	20	11.4
4	2-Fluorobenzyl alcohol	DB1701-30m	Method A	20	12.8
5	4-Chlorobenzyl alcohol	DB1701-30m	Method A	20	15.1
6	3-Chlorobenzyl alcohol	DB1701-30m	Method A	20	15.2
7	2-Chlorobenzyl alcohol	DB1701-30m	Method A	20	15.8
8	4-Bromobenzyl alcohol	DB1701-30m	Method A	20	17.1
9	3-Bromobenzyl alcohol	DB1701-30m	Method A	20	17.2
10	2-Bromobenzyl alcohol	DB1701-30m	Method A	20	17.7
11	4-Methylbenzyl alcohol	DB1701-30m	Method A	20	14.3
12	3-Methylbenzyl alcohol	DB1701-30m	Method A	20	14.0
13	2-Methylbenzyl alcohol	DB1701-30m	Method A	20	13.4
14	4-Methoxybenzyl alcohol	HP-5	Method C	20	10.9
15	3,4-Dimethoxybenzyl alcohol	HP-5	Method D	30	n.d.
22	*trans*-Cinnamic alcohol	HP-5	Method C	20	11.0

HPLC

HPLC measurements were performed on a Prominence-i LC 2030C 3D (Shimadzu) with a Shimadzu Shim-pack GIST 5 µm C18 AQ (150 mm length, 4.6 mm inner diameter) column.

Method E: Flow: 1 ml min^{-1}; oven temperature: 30 °C; gradient: 40% B in 20 min, 5 min hold; gradient: 0% B in 5 min, 15 min hold. Analysis at λ of 254 nm.

Mobile phase: A: 50 mM Ammonium formate buffer pH 9; B: Methanol + 0.1% TFA. Retention time of IS (acetophenone): 27.2 min; retention times of carboxylic acids are listed in Table 15.

Table 4.15. List of retention times and analysis details of HPLC quantification of pyridine carboxylic acids.

Entry	Substrate	Method	Sample volume (µl)	Carboxylic acid (**e**) (min)
17	4-Pyridinemethanol	Method E	4	3.4
18	3-Pyridinemethanol	Method E	4	4.3
19	2-Pyridinemethanol	Method E	4	3.6

4.4.15.3 GC-MS analysis for nitrile formation

GC-MS measurements were done on a QP2010SE GCMS system (Shimadzu) using an Agilent J&W DB1701 (30 m, 250 µm, 0.25 µm) column with He as carrier gas.

Method: T injector 250 °C; constant pressure 6.9 psi; temperature program: 80 °C, hold 6.5 min; 10 °C min^{-1} to 160 °C, hold 5 min; 20 °C min^{-1} to 200 °C, hold 2 min; 20 °C min^{-1} to 280 °C, hold 1 min. MS program, parameters: Ion Source Temperature: 200 °C, Detector Voltage: 0.1 kV, Start Time: 2 min, End Time: 28.5 min, Start m/z: 43, End m/z: 600.

4.5 References

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