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Catalytic Promiscuity of Galactose Oxidase: A Mild Synthesis of Nitriles from Alcohols, Air and Ammonia

Jan Vilím[a], Tanja Knaus[a], and Francesco G. Mutti*[a]

Dedication. ((optional))

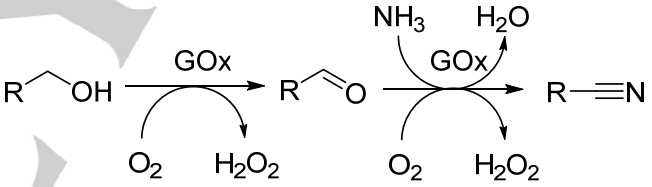
Abstract: *We present an unprecedented catalytic promiscuous activity of the copper-dependent enzyme, galactose oxidase. The enzyme catalyses the one-pot conversion of alcohols into the related nitriles under mild reaction conditions in ammonium buffer, consuming ammonia as the source of nitrogen and dioxygen (from air at atmospheric pressure) as the only oxidant. Thus, this green method does not require either cyanide salts, or toxic metals, or undesired oxidants in stoichiometric amounts. The substrate scope of the reaction includes benzyl and cinnamyl alcohols as well as 4- and 3-pyridylmethanol, giving access to valuable chemical compounds. The oxidation proceeds via oxidation from alcohol to aldehyde, in situ imine formation and final direct oxidation to nitrile.*

Catalytic enzyme promiscuity is the ability of an enzyme to catalyse chemical reactions that are different from the natural one.[1] Even after two decades of intensive investigations, new notable cases of catalytic enzyme promiscuity have been recently revealed and applied in chemical synthesis [2] as well as in synthetic biology.[3] Herein, we report an unprecedented catalytic promiscuity of a galactose oxidase, which is the conversion of selected alcohols into nitriles.

General methods for the synthesis of nitriles include dehydration of amides,[4] formal acid-nitrile exchange,[5] Sandmeyer and Rosenmund-von Braun reactions,[6] transition-metal catalyzed cyanation,[7] electrophilic cyanide transfer[8] and radical-type cleavage reactions.[9] 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)[10], or amines (in presence of metal catalysts or catalytic TEMPO or stoichiometric oxidants),[11] or azides,[12] or pre-formed oximes,[13] or organic halides,[14] or arenes.[15] Benzonitriles are also produced on industrial scale from toluene by ammoxidation using heterogeneous catalysts, ammonia and dioxygen (450 °C, 2 bar).[16] The direct conversion of alcohols into nitriles attracts interest, but it requires a metal and/or an organic catalyst in presence of supra-stoichiometric amounts of an organic oxidant and ammonium species.[17] 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, making use of Cu(II) at high temperature or Fe(III)/TEMPO in MeCN.[18]

Biocatalytic approaches enable synthesis of nitriles under mild reaction conditions. Those methods include the use of aldoxime dehydratases,[19] hydroxynitrile lyases (i.e. addition of cyanide to carbonyl compounds),[20] halohydrin dehalogenases (i.e. ring-opening of epoxides by cyanide),[21] and amine oxidases in combination with cyanide salt.[22] Other enzyme families such as nitrile synthetase,[23] β-cyano-L-alanine synthase[24] and cytochromes [25] have limited synthetic applicability. However, there is no report about a one-enzyme conversion of alcohols into nitriles.

Surprisingly, during the oxidation of benzyl alcohol (**1a**, 10 mM) to benzaldehyde (**1b**) in ammonium formate buffer (600 mM, pH 9) catalysed by purified Strep-tagged galactose oxidase (GOx, 25 μM) from *Fusarium sp*. $M_{3\text{-}5}$,[26] we noticed the unexpected formation of just 1.2% of benzonitrile (**1c**) that sparked our interest (Scheme 1).



Scheme 1. Conversion of alcohols to nitriles catalysed 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 stabilisation of its holo-form for biocatalytic reactions *in vitro*.[27] 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.[26f] However, the use of phosphate buffer poses the issue of precipitation of nearly insoluble copper phosphate.[28] 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). Fig 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, SI 4.1). Switching from Tris-HCl to $HCOONH_4$ buffer resulted in a similar trend albeit nitrile **1c** was formed along with **1b**. Thus, 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

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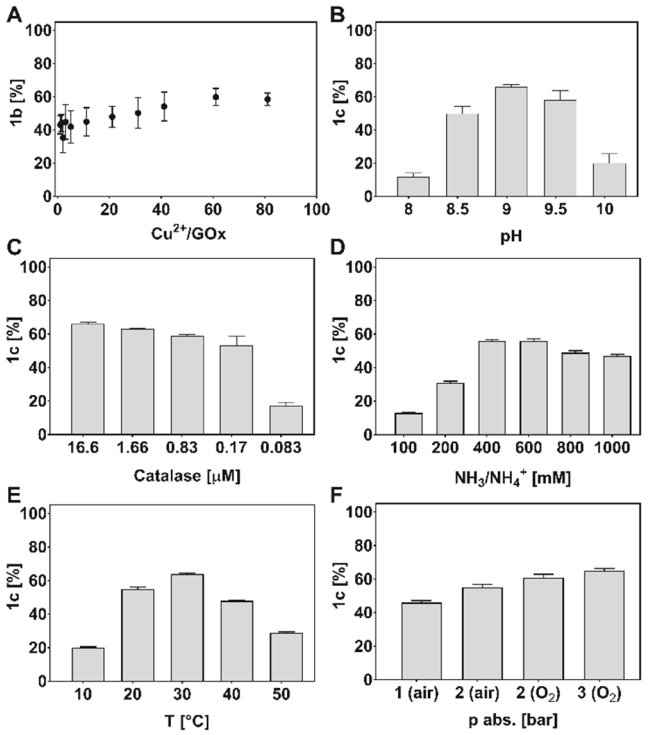

Figure 1. Optimization of reaction conditions and determination of substrate scope. A) Influence of copper B) Influence of pH C) Optimal amount of catalase D) Concentration of ammonium species E) Influence of temperature F) Supplementation of oxygen.

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.[26f] 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 1B). A second set of experiments aimed at minimising the amount of catalase for the transformation of **1a** (10 mM) to **1c** at pH 9. Fig. 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. 1D and 1E). After the optimisation 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.[26c, 26f, 27a, 27c]. Interestingly, the supplementation of O_2 as pressurised air or pure O_2 increased slightly the yield of **1c** (Fig. 1F). However, a large-scale biocatalytic conversion of alcohols to nitriles operating under pressure would have the disadvantage of consuming energy for pressurisation of the system. Thus, further optimisation was conducted using air at atmospheric pressure.

The work with highly purified GOx was crucial for demonstrating the promiscuous formation of nitriles from alcohols (Figure S3). 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.[29] Hence, we tested GOx as *E. coli* cell free extract (CFE) because costly and time-consuming purification steps are avoided [26f] and, possibly, higher GOx activity may be retained. Indeed, optimisation of the reaction conditions for the conversion of **1a** to **1c** using CFE permitted to increase the TON up to ca. 3300 (Fig. 2B), which is a value already suitable for a large scale application.[29] Yields of **1c** were in line with the experiments using purified GOx (Fig. 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

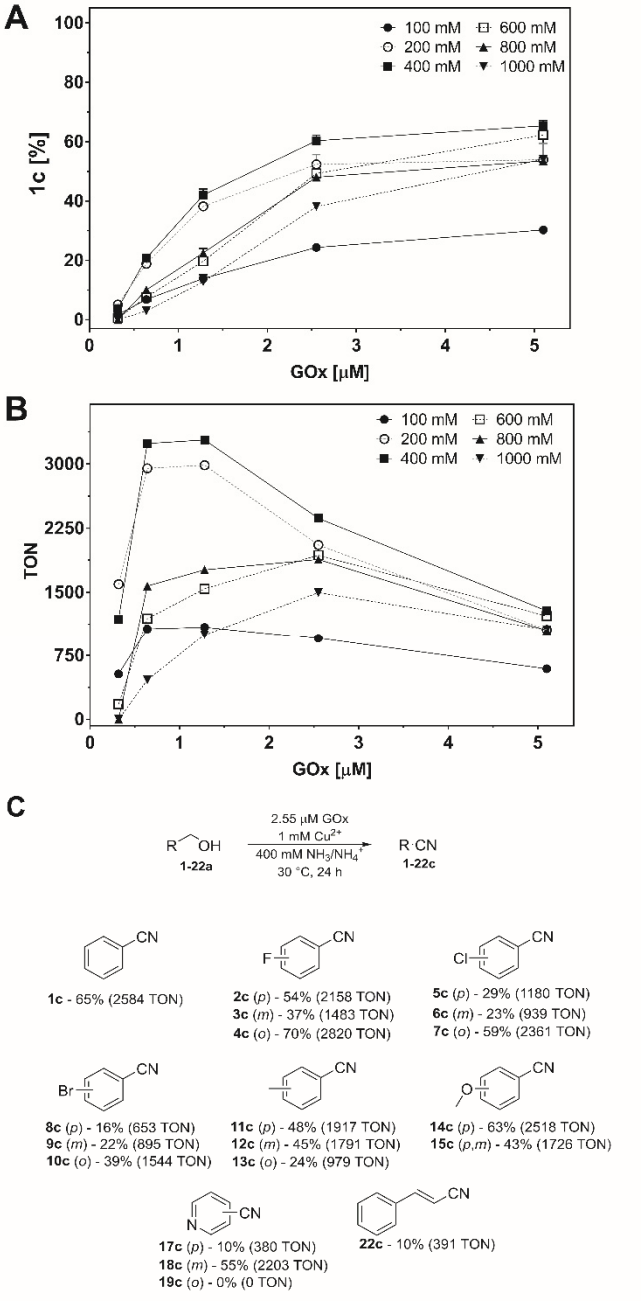

Figure 2. Synthesis of nitriles from alcohols using CFE. Analytical yield of **1c** (A) and TON (B) at varied concentration of NH_3/NH_4^+ and GOx. Substrate scope (C). For experimental details, see SI.

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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 optimised 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. 2C, SI 4.8).

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 catalysed by GOx.[30] 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 nitrile (and the chemoselectivity 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 optimised reaction conditions using CFE. After 24 h, the reaction afforded >99% analytical yield into nitrile **4c** (exactly quantified with internal standard). After extraction 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** in 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.[17a, 31] A series of reactions (Table 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.[26f] 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). In the same way, formation of **1c** from **1a** was observed only in presence of GOx. Finally, the transformation of aldehyde to nitrile may occur via two possible paths: 1) imine formation by reaction with ammonia and subsequent promiscuous oxidation to nitrile via hydride abstraction, or 2) imine formation, subsequent promiscuous hydroxylation to oxime and final dehydration to nitrile. Thus, benzaldehyde oxime (**1d**) was incubated with GOx under various conditions (Table 1 entries 7-10; for details Table S11) but dehydration to nitrile was never observed. Consequently, nitrile **1c** is formed by direct oxidation of the imine intermediate.

Entry	Substrate	pH	GOx (μM)	Albumine (μM)	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

Table 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.

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 to recently reported approaches used to transform alcohols or aldehydes into nitriles, [7a, 10a, 10e, 17] the GOx-catalysed 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,[32] whereas benzonitriles constitute the active core of the large majority of nitrile-containing pharmaceuticals.[33] Moreover, 3-cyanopyridine is a precursor to vitamin B3, to which it can be converted by established enzymatic methods.[34] 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.

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Keywords: biocatalysis • enzyme promiscuity • alcohol oxidation • nitriles • copper oxidases

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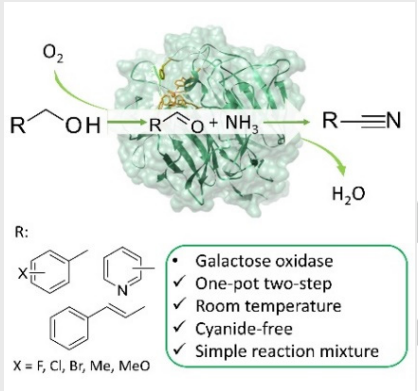
Unprecedented catalytic promiscuity of copper dependent alcohol oxidase allows one-pot transformation of selected alcohols into nitriles under mild, cyanide-free conditions using ammonia as nitrogen donor and dioxygen as oxidant. This reaction provides a valuable addition to enzymatic methods for nitrile synthesis, since direct enzymatic transformation of alcohol to nitriles has been unknown so far.



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Catalytic Promiscuity of Galactose Oxidase: A Mild Synthesis of Nitriles from Alcohols, Air and Ammonia

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