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

*Jan Vilím, Tanja Knaus, and Francesco G. Mutti**

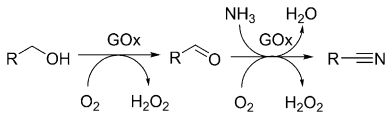
Abstract: *We report an unprecedented catalytically promiscuous activity of the copper-dependent enzyme galactose oxidase. The enzyme catalyses the one-pot conversion of alcohols into the related nitriles under mild reaction conditions in ammonium buffer, consuming ammonia as the source of nitrogen and dioxygen (from air at atmospheric pressure) as the only oxidant. Thus, this green method does not require either cyanide salts, 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 through 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 catalytically promiscuous activity of a galactose oxidase, namely 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-catalysed 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] amines (in presence of metal catalysts or catalytic TEMPO or stoichiometric oxidants),[11] azides,[12] pre-formed oximes,[13] organic halides,[14] or arenes.[15] Benzonitriles are also produced on the 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 the presence of superstoichiometric amounts of an organic oxidant and ammonium species.[17] However, replacing chemical oxidants with dioxygen would significantly increase the atom-efficiency and the environmental footprint of the reaction. A few systems for the aerobic conversion of alcohols into nitriles have been published that make use of Cu^{II} at high temperature or Fe^{III}/TEMPO in MeCN.[18]

Biocatalytic approaches enable the synthesis of nitriles under mild reaction conditions. Those methods include the use of aldoxime dehydratases,[19] hydroxynitrile lyases (addition of cyanide to carbonyl compounds),[20] halohydrin dehalogenases (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 1.2 % of benzonitrile (**1c**), which sparked our interest (Scheme 1).



Scheme 1. Conversion of alcohols into nitriles catalysed by a 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+} on 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 the 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). Figure 1 A shows that the conversion of **1a** (10 mM) into **1b**, measured after 40 min, rose progressively at increasing ratios of Cu^{2+} to purified GOx (2.5 μM). The highest yield was observed at a molar Cu^{2+}/GOx ratio of approximately 60:1 (for details, see Section S4.1 in the

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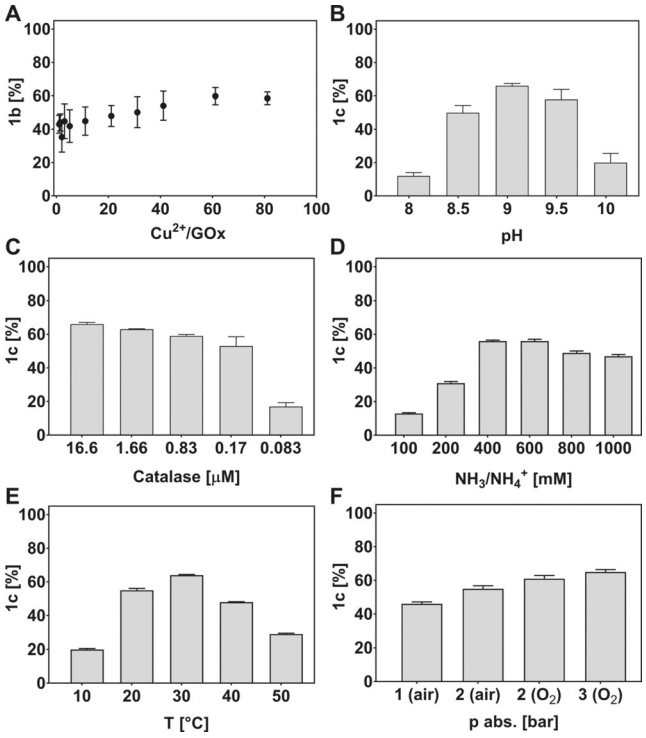

Figure 1. Optimisation of reaction conditions and determination of substrate scope. A) Influence of copper. B) Influence of pH. C) Optimal amount of catalase. D) Concentration of ammonium species. E) Influence of temperature. F) Supplementation of oxygen.

Supporting Information). Switching from Tris-HCl to $HCOONH_4$ buffer resulted in a similar trend, although nitrile **1c** was formed along with **1b**. A 50:1 molar ratio of Cu^{2+}/GOx was used for the continuation of our study. We then investigated the influence of the pH on 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) have not been reported, 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. Figure 1C shows that the addition of catalase positively affected the reaction albeit a minimal concentration of 0.83 μM (equal to 0.05 mg mL^{-1}) was sufficient. Evaluation of the influence of the concentration of ammonium species and temperature on the yield of **1c** showed maxima in the range of 400–600 mM of NH_3/NH_4^+ and at 30 °C (Figure 1D and 1E). After optimisation of the reaction parameters, we investigated the influence of air and pure dioxygen (even under pressure) on the progress of the reaction, since dioxygen is the oxidant in the GOx catalytic cycle.[26c,f,27a,c] Interestingly, supplementation of O_2 as pressurised air or pure O_2 slightly increased the yield of **1c** (Figure 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.

Working 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 around 230, which is insufficient for synthetic application with oxidoreductases.[29] Hence, we tested GOx as a *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** into **1c** using CFE permitted an increase in the TON to up to around 3300 (Figure 2B), which is a value suitable for large-scale applications.[29] Yields of **1c** were in line with the experiments using purified GOx (Figure 2A). In particular, the highest TON of around 3300 (1.28 μM GOx as CFE) correlated to a 42% yield of **1c**, whereas the highest yield of 65% (2.55 μM GOx as CFE) correlated to a TON of around 2600. Using a 2.55 μM GOx loading as a 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). 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 and TONs, see Figure 2C and Section S4.8 in the Supporting Information).

Interestingly, within a homologous series, benzyl alcohols containing electron-withdrawing substituents in *ortho* positions were converted with higher yields (**4c**, **7c**, **10c**) compared to the *para*-substituted (**2c**, **5c**, **8c**) and especially the *meta*-substituted (**3c**, **6c**, **9c**) ones. The effect was reversed with the electron-donating methyl substituent, since *ortho*-methyl benzyl alcohol (**13c**) was converted to a lesser degree 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 a 10% yield of the related nitrile **22c**. Moreover, 4-pyridyl methanol and 3-pyridyl methanol were also transformed into the corresponding nitriles (**17c**, **18c**) in 10% and 55% yield, respectively. Besides the formation of the nitrile products, variable amounts of carboxylic acids (**1**–**14e**, **17**–**18e**, **22e**) were detected, which is 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) or 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 yields of the nitriles (and the chemoselectivity of the reaction) were somehow dependent on the scale of the reaction. For instance, a preparative-scale synthesis was

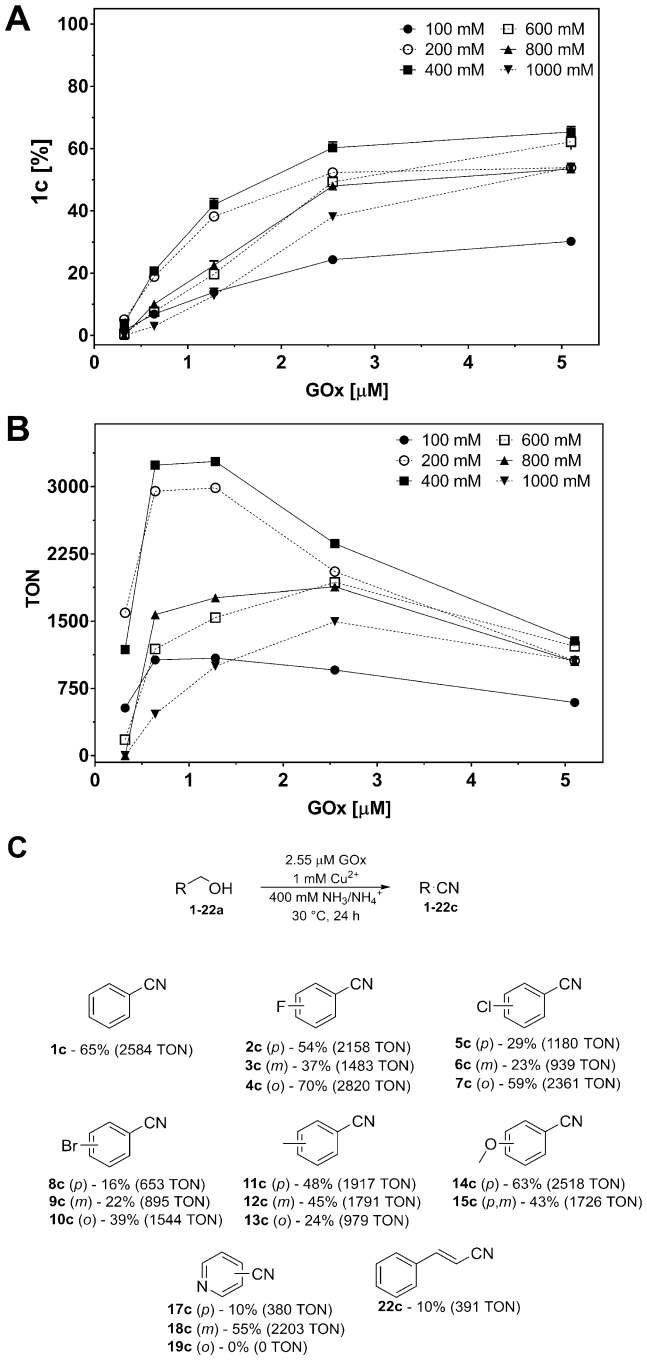

Figure 2. Synthesis of nitriles from alcohols using a CFE from *E. coli* cells (over)expressing GOx. Analytical yield of **1c** (A) and TON (B) at varied concentrations of NH_3/NH_4^+ and GOx. C Substrate scope. For experimental details, see the Supporting Information.

performed with **4a** (151 mg, 1.2 mmol) under the optimised reaction conditions using a CFE. After 24 h, the reaction afforded >99% analytical yield of nitrile **4c** (exactly quantified with internal standard). After extraction and solvent evaporation, nitrile **4c** was isolated in 75% yield and in pure form (no further purification step was required). Conversely, the biocatalytic conversion of **4a** on an analytical scale (Figure 2C) produced a 70% analytical yield of nitrile **4c** and 5% carboxylic acid **4e**. We attribute the discrepancy to different aeration and agitation between analytical-scale and preparative-scale reactions.

Regarding the mechanism of formation of the nitrile from the alcohol, we further confirmed the promiscuous activity of GOx by exploring a possible non-enzymatic or non-specific conversion of the aldehyde **1b** into nitrile **1c**. There are reports describing how H_2O_2, Cu^{2+}, or formate may contribute to the conversion of **1b** into **1c** (and derivatives thereof), but in the presence of additional reagents and under particular reaction conditions.[17a,31] However, series of reactions (Table 1) revealed that nitrile **1c** is indeed produced

Table 1: Study on the formation of nitriles from **1b** or **1d** using GOx or albumin.[a]

Entry	Substrate	pH	GOx (μM)	Albumin (μM)	Yield (%)
1	**1b**	9	20	–	47
2	**1b**[b]	9	20	–	33
3	**1b**	9	–	–	0
4	**1b**[b]	9	–	–	<0.5
5	**1b**	9	–	20	0
6	**1b**[b]	9	–	20	<0.5
7	**1d**	7	20	–	0
8	**1d**	9	20	–	0
9	**1d**[c]	7	20	–	0
10	**1d**[c]	9	20	–	0

[a] Unless stated otherwise, Cu^{2+} (50 equiv) was added. For details, see the Supporting Information. [b] H_2O_2 (10 mM) was added. [c] Cu^{2+} was omitted.

from aldehyde **1b** only in the 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), thus confirming the detrimental effect of H_2O_2 at high concentration.[26f] Several control experiments, including reactions with 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 the aldehyde into the nitrile may occur through two possible pathways: 1) imine formation through reaction with ammonia and subsequent promiscuous oxidation to the nitrile through hydride abstraction, or 2) imine formation, subsequent promiscuous hydroxylation to an oxime, and final dehydration to the nitrile. To investigate this, benzaldehyde oxime (**1d**) was incubated with GOx under various conditions (Table 1 entries 7–10; for details Table S11) but dehydration to the nitrile was never observed. Consequently, nitrile **1c** is formed through direct oxidation of the imine intermediate.

In conclusion, we have discovered a new promiscuous activity of the galactose oxidase that enabled the one-pot synthesis of benzyl, pyridyl, and cinnamyl nitriles from the corresponding alcohols using only ammonia as a source of nitrogen and dioxygen as an innocuous oxidant. Compared to recently reported approaches used to transform alcohols or aldehydes into nitriles,[7a,10a,e,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 a CFE increased the TON to synthetically applicable levels

and avoided any purification steps. This promiscuous activity of GOx could have notable applications since cinnamonitrile is an important synthetic aroma,[32] and benzonitriles constitute the active core of the large majority of nitrile-containing pharmaceuticals.[33] Moreover, 3-cyanopyridine is a precursor to vitamin B3, into which it can be converted through established enzymatic methods.[34] Future research will focus on searching for other promiscuous copper-dependent alcohol oxidases that are active on structurally different alcohols, in order to enable even broader application of this new biocatalytic reaction.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: alcohol oxidation · biocatalysis · catalytic promiscuity · copper oxidases · nitriles

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