Enzymatic Reduction of Levoglucosenone by an Alkene Reductase (OYE 2.6): a Sustainable Metal- and Dihydrogen-free Access to the Green Solvent Cyrene®

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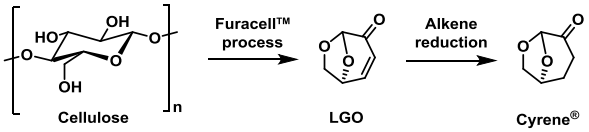
Levoglucosenone (**LGO**) has been successfully converted into the green polar aprotic solvent **2H-LGO** (aka Cyrene®) through an enzymatic process involving alkene reductases: wild-type Old Yellow Enzyme 2.6 (OYE 2.6 *wt.*) from *Pichia stipidis* and its mutant that present the best conversion rates (OYE 2.6 $Tyr^{78}Trp$). This enzymatic process has been optimized in order to avoid the formation of the side-product (1*S*,2*R*)-2-hydroxy-6,8-dioxabicyclo[3.2.1]octan-4-one (**OH-LGO**) and reach total conversion (99%). Cyrene® was then succesfully isolated by continuous extraction in quantitative yields (99%).

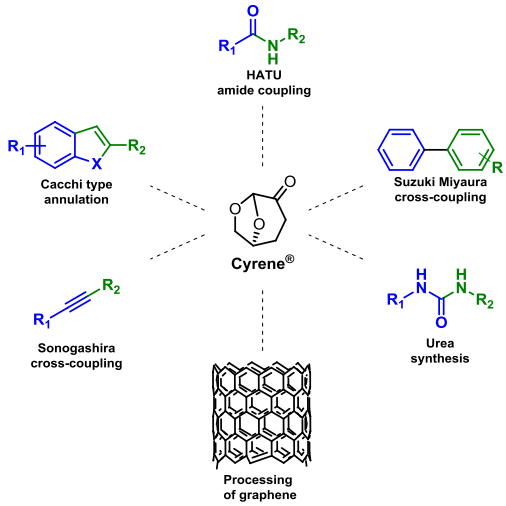
Introduction

2H-LGO (Cyrene®) is a highly valuable renewable chemical that can be obtained from the reduction of the alkene moiety of Levoglucosenone (**LGO**), a relatively complex chiral chemical platform derived from the catalytic aerobic pyrolysis (CFP), such as Furacell™ process, of cellulose and hemicellulose (Scheme 1).[1, 2]



Scheme 1. Synthetic route to cellulose-based Cyrene®

Cyrene® has been recognized as a safe and promising substituent for toxic dipolar aprotic solvent, such as NMP, DMF or sulpholane,[3] and many examples of its successful utilization in a wide variety of organic reactions,[4] such as amide coupling,[5] Suzuki-Miyaura cross-coupling,[6] synthesis of ureas,[7] dispersion of graphene,[8] Sonogashira cross-coupling and Cacchi-type annulation[9] and metal-org frameworks (MOF) synthesis,[10] have been described in the literature (Scheme 2). Furthermore, Cyrene® can be used as building block for the synthesis of compounds of interest, such as **2H-LGO**-based oximes[11] or (S)-γ-hydroxymethyl-α,β-butyrolactone,[12, 13] a known precursor of dairy lactone.All the methods described in the literature to access Cyrene® involve a metal catalyst and dihydrogen.[13-24] Despite being efficient in terms of yields and cost, not only these methods can be dangerous to perform - notably at large scale - (i.e., dihydrogen), but the presence of potential metal residues - even in ppm quantities - in Cyrene® may also limit its utilization in certain applications.[25-27] These drawbacks could thus limit the potential of Cyrene®, specifically in the food/feed, cosmetic and pharmaceutical sectors.



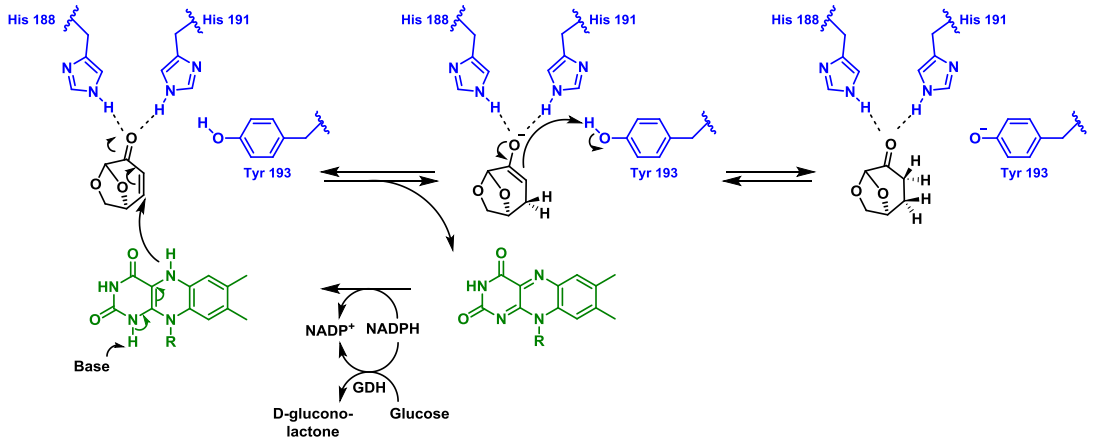
Scheme 2. The different applications of Cyrene® as green solvent

In order to offer a greener method to access Cyrene®, the use of an alkene reductase, the Old Yellow Enzyme 2.6 (OYE 2.6) from *Acinetobacter sp.*, seemed to be a good alternative to the classical methods. Indeed, OYEs are Flavin-dependent enzymes that catalyze the reduction of α,β-unsaturated ketones. The substrate diversity of this enzyme is relatively wide, accepting

compounds such as carvone, pulegone, 2-methyl-2-cyclopentenone, 3-methylfuran-2(5H)-one and other analogs with more or less bulky groups.[28] The proven flexibility of OYE 2.6 allowed us to prognosticate a good activity in regard to **LGO**.

Results and Discussion

The first step of this study dealt with the determination of the activity of OYE 2.6 toward **LGO**. All OYEs appear to follow a ping-pong mechanism where the first step is the reduction of the tightly bound Flavin Mononucleotide (FMN) cofactor by Nicotinamide Adenine Dinucleotide Phosphate (NADPH). The resulting $NADP^+$ leaves the active site, allowing access for the substrate to enter. In the case of OYE 2.6, the α,β-unsaturated ketone, stabilized by two histidine residues, undergoes reduction to the corresponding saturated product which in turn dissociates to concede its place to another NADPH molecule in order for the catalytic cycle to continue.[29] The enzymatic reaction was first tested with OYE 2.6 $Tyr^{78}Trp$, the most known active mutant of this enzyme.[28] The original procedure consisted in the addition of OYE 2.6 $Tyr^{78}Trp$ (50 µg) to 1 mL of a 10 mM **LGO** in phosphate buffer (pH 8.0) complemented with a cofactor regeneration system composed of Glucose Dehydrogenase (GDH), $NADP^+$ and glucose (Scheme 3). Monitoring of the reaction using GC/MS revealed the presence of the target (i.e., Cyrene®) alongside a side-product (Figure 1). Unfortunately, mass analysis did not allow the identification of it structure.



Scheme 3. Alkene reduction of Levoglucosenone into Cyrene® using OYE 2.6 (Proposed mechanism)

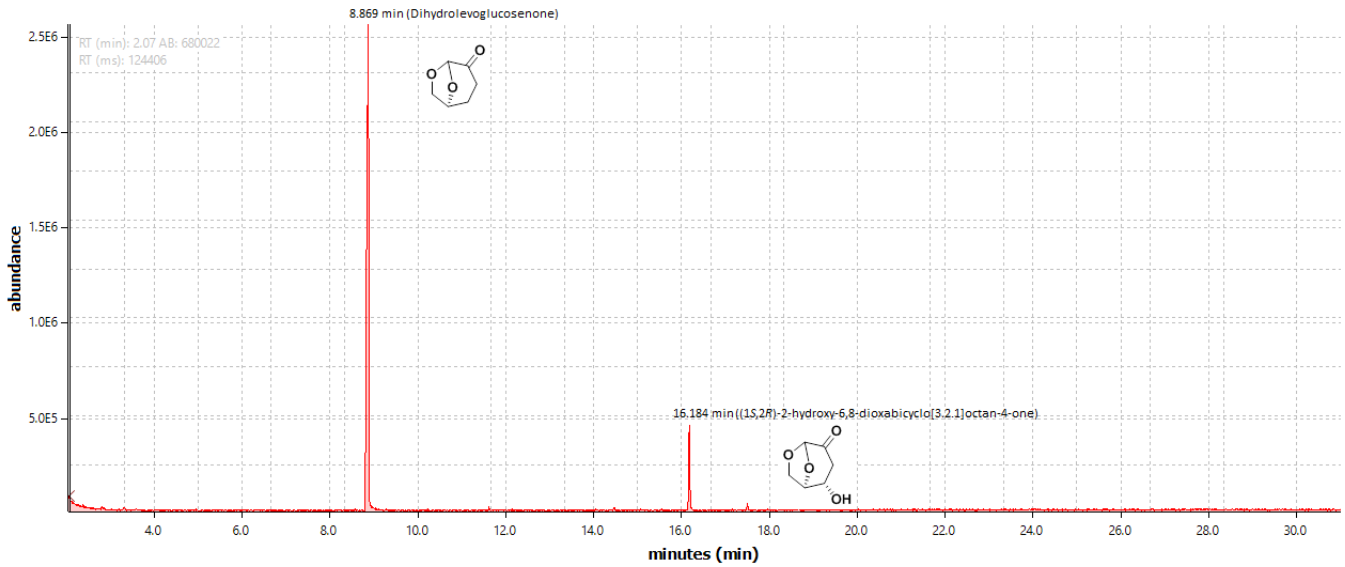
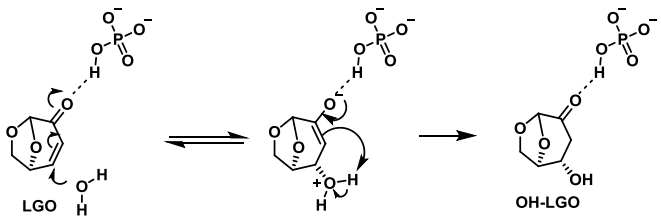


Figure 1. GC-FID analysis of non-optimized reaction conditions using OYE 26 $Tyr^{78}Trp$

By running a negative control without OYE 2.6 $Tyr^{78}Trp$, the formation of the unwanted side-product was still observed. Our first assumption was that GDH reduced the ketone moiety of **LGO** into the corresponding alcohol. Yet, a second negative control in the absence of both OYE 2.6 $Tyr^{78}Trp$ and GDH resulted in the same results, proving our hypothesis wrong. In view of these results, it became necessary to scale up the negative control in order to isolate a sufficient amount of the side-product to perform in-depth NMR analysis. To do so, continuous extraction of a reaction mixture composed of 10 mM **LGO** in a final volume

of 500 mL phosphate buffer was incubated overnight at room temperature. Multidimensional NMR analysis of the crude mixture was performed and the side-product proved to be enantiopure (1*S*,2*R*)-2-hydroxy-6,8-dioxabicyclo[3.2.1]octan-4-one (**OH-LGO**) (Figure 2), obtained through the phosphate-catalyzed Michael addition of water to **LGO** as already described in the literature (Scheme 4).[30-32]



Scheme 4. Michael addition of water on **LGO** in phosphate buffer

To overcome this side-reaction, as the hydration of **LGO** is a relatively slow process,[32] dropwise addition of a solution of **LGO** in ethanol to the reaction mixture containing OYE 2.6 Tyr[78]Trp, NADP+, GDH and glucose in phosphate buffer (pH 8.0) was attempted. We were delighted to observe that this procedure resulted in the total conversion of **LGO** into Cyrene® without any trace of the side product **OH-LGO**. Continuous extraction using ethyl acetate allowed us to isolate Cyrene® in quantitative yield (99%). This extraction step using ethyl acetate is unfortunately necessary considering the working concentration and volume.

Our objective being to offer a synthetic procedure compatible for uses of Cyrene® in the food/feed, cosmetic and pharmaceutical industries, the same assay was performed using wild-type OYE 2.6 (OYE 2.6 *wt.*) to check whether an unmodified enzyme could be used. Here again, total conversion (99%) of **LGO** into Cyrene® and absence of any **OH-LGO** traces were observed.

In summary, the proof-of-concept of the preparation of Cyrene® from **LGO** through an OYE 2.6-mediated biocatalytic process in water has been validated. However, the current procedure presents two major drawbacks to be economically or industrially relevant for multi-kilo production of Cyrene®. The first one is the low concentration used. One solution to overcome it would be the continuous extraction of Cyrene® from the reaction medium using membrane contactor.[33, 34] Indeed, this membrane-based technique consisting in the continuous extraction of Cyrene® while avoiding the contact of the extracting phase (i.e., organic solvent, here ethyl acetate) with the enzyme should not only improve the productivity but also preserve enzyme activity. The second drawback is the use of glucose as hydride donor. Although this is a very convenient way to regenerate NADPH on small scale for proof-of-concept, other enzymatic and non-enzymatic alternatives, such as the ones described by Wang et al.[35] can be used for industrial scale synthesis.

Conclusion

In this paper, a low toxicity and greener method to access Cyrene® has been developed and optimized from Levoglucosenone (**LGO**) using biocatalysis. The use of an alkene reductase from *Acinetobacter sp.* (OYE 2.6) allows to reach total conversion of the substrate into Cyrene® while avoiding the formation of a byproduct, (1*S*,2*R*)-2-hydroxy-6,8-dioxabicyclo[3.2.1]octan-4-one (**OH-LGO**). In an effort to develop a multi-kilo route to Cyrene® based on this biocatalytic alkene reduction, the use of industrially relevant NADPH regeneration systems and a new purification method involving membrane-contactor will be investigated and reported in due course.

Acknowledgements

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Experimental Section

General: Evaporations were conducted under reduced pressure at temperatures below 35 °C unless otherwise noted. ^{1}H NMR spectra were recorded at 300 MHz at 25 °C in the indicated solvent and referenced to residual protons ($CDCl_3$, 7.26 ppm; CD_3OD, 4.87 ppm; D_2O, 4.75 ppm). ^{13}C NMR spectra were recorded at 75 MHz at 25 °C in the indicated solvent and referenced to solvent ($CDCl_3$, 77.2 ppm; CD_3OD, 49.2 ppm).

GC/MS methods: Enzymatic reactions were extracted with EtOAc and 0.01% methyl benzoate was added as an internal standard). A 1.0 µL portion of the organic layer was used for GC/MS analysis (EI) equipped with a DB-17 column (0.25 mm × 30

m, 0.25 µm film thickness) using the following conditions: 60 °C (2 min) to 250 °C (15 min) at 10 °C.min^{-1}. These conditions allowed baseline separation of all relevant analytes in this study (Methyl-benzoate, t_r = 7.89 min; **LGO**, t_r = 7.08 min; **HBO**, t_r = 10.91 min; **OH-LGO**, t_r = 16.18 min; **Cyrene®**, t_r = 8.86 min).

Synthesis of (1R)-6,8-dioxabicyclo[3.2.1]octan-4-one (2H-LGO): 10% Pd/C (10% w/w, 100 mg) was added to a solution of **LGO** (1 g, 7.96 mmol) in EtOAc (40 mL, 0.2 M) at rt. The stirred suspension was degassed 3 times and kept under nitrogen. The suspension was then hydrogenated under a hydrogen atmosphere at room temperature until TLC showed complete consumption of the starting material. The crude mixture was filtered over a pad of Celite and the filtrate was concentrated to dryness with silica gel. The crude product was purified by silica gel chromatography (elution with 10 to 60% EtOAc in cyclohexane) to yield pure **2H-LGO** (colorless oil, 88 mg, 87%). ^{1}H and ^{13}C NMR data matched those reported by Allais *et al.*[20]

Overexpression and Purification of OYE 2.6 (*wt.* and Tyr^{78}Trp) Crude Lysate from *Acinetobacter sp.*: The plasmids that overexpressed OYE 2.6 (*wt.* and Tyr^{78}Trp) were used to transform *E. coli* BL21(DE3) cells with selection for ampicillin resistance (100 µg/mL) on LB plates. Single colonies of the strains were used to inoculate 12 mL portions of liquid LB medium containing 50 µg/mL ampicillin and the culture was grown overnight at 37 °C and 250 rpm. An aliquot (10 mL) was diluted into 1 L of the same medium in a 2 L baffled flask. The culture was grown at 37 °C with stirring at 250 rpm until reaching an optical density of 0.6 at 600 nm. Overexpression of OYE 2.6 was induced by adding sterile isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The culture was incubated for an additional 4 h at 30 °C, then the cells were harvested by centrifugation (6000 × *g* at 4 °C for 15 min). The cell paste was resuspended in 6 mL of cold lysis buffer (50 mM Tris-HCl, pH 8.0) and disrupted by two passages through a French pressure cell at 18,000 psi. The lysate was clarified by centrifugation (18,000 × *g* for 20 min at 4 °C). The resulting supernatant was passed through 10 mL of glutathione agarose (Clontech) using an FPLC system (Pharmacia) with 1 × PBS buffer as the mobile phase. Once the A280 returned to a baseline reading, the desired protein was eluted by adding 10 mL of reduced glutathione (10 mM, freshly prepared) in Tris−HCl (50 mM, pH 8.0), and then the appropriate fractions were concentrated to ∼20−40 mg/mL by ultrafiltration (Amicon Ultra-4 membrane, 10 000 NMWL). An equal volume of glycerol was added prior to storage at -20 °C.

Enzymatic Assay for the Alkene Reduction of LGO Using Purified OYE 2.6 Tyr^{78}Trp: To a solution containing 10 mM **LGO**, 2.5 U of GDH, 200 mM glucose and 0.3 mM $NADP^+$ in 1 mL Na_2PO_4 buffer (100 mM) was added 50 µg of purified OYE 2.6 Tyr^{78}Trp and the solution was incubated at rt overnight. The reaction mixture was then centrifuged at 12,000 rpm / 20 °C for 2 minutes and the supernatant was extracted with EtOAc that also contained 0.01% methyl benzoate as an internal standard. The organic layer was analyzed by GC/MS.

Enzymatic Assay for the Alkene Reduction of LGO Using Purified OYE 2.6 *wt.*: To a solution containing 10 mM **LGO**, 2.5 U of GDH, 200 mM glucose and 0.3 mM $NADP^+$ in 1 mL Na_2PO_4 buffer (100 mM) was added 50 µg of purified OYE 2.6 *wt.* and the solution was incubated at rt overnight. The reaction mixture was then centrifuged at 12,000 rpm / 20 °C for 2 minutes and the supernatant was extracted with EtOAc that also contained 0.01% methyl benzoate as an internal standard. The organic layer was analyzed by GC/MS.

Large Scale Alkene Reduction of LGO into Cyrene® Using Purified OYE 2.6 Tyr^{78}Trp: To a solution containing 125 U of GDH, 200 mM glucose, 0.3 mM $NADP^+$ and 50 µg of purified OYE 2.6 Tyr^{78}Trp in 50 mL Na_2PO_4 buffer (100 mM) was added dropwise 60 µL of **LGO** dissolved in 1.5 mL EtOH over 6 hours. The reaction mixture was submitted to continuous extraction overnight using EtOAc. The organic layer was directly analyzed by GC/MS to confirm the completion of the reaction. The organic layer was evaporated *in vacuo* to yield pure **Cyrene®**.

Notes and references

1. F. Shafizadeh, Y. Z. Lai and C. R. McIntyre, *J. Appl. Polym. Sci.*, 1978, **22**, 1183-1193.
2. G. R. Court, C. H. Lawrence, W. D. Raverty and A. J. Ducan, US20120111714, 2011.
3. J. Sherwood, M. de Bruyn, A. Constantinou, L. Moity, C. R. McElroy, T. J. Farmer, T. Duncan, W. Raverty, A. J. Hunt and J. H. Clark, *Chem. Commun.* , 2014, **50**, 9650-9652.
4. J. E. Camp, *ChemSusChem*, 2018, **11**, 3048-3055.
5. K. L. Wilson, J. Murray, C. Jamieson and A. J. B. Watson, *Org. Biomol. Chem.*, 2018, **16**, 2851-2854.
6. K. L. Wilson, J. Murray, C. Jamieson and A. J. B. Watson, *Synlett*, 2018, **29**, 650-654.
7. L. Mistry, K. Mapesa, T. W. Bousfield and J. E. Camp, *Green Chem.*, 2017, **19**, 2123-2128.
8. H. J. Salavagione, J. Sherwood, M. De Bruyn, V. L. Budarin, G. J. Ellis, J. H. Clark and P. S. Shuttleworth, *Green Chem.*, 2017, **19**, 2550-2560.
9. K. L. Wilson, A. R. Kennedy, J. Murray, B. Greatex, C. Jamieson and A. J. B. Watson, *Beilstein J. Org. Chem.*, 2016, **12**, 2005-2011.
10. J. Zhang, G. B. White, M. D. Ryan, A. J. Hunt and M. J. Katz, *ACS Sustain. Chem. Eng.*, 2016, **4**, 7186-7192.
11. A. Alhifthi, B. L. Harris, L. Goerigk, J. M. White and S. J. Williams, *Org. Biomol. Chem.*, 2017, **15**, 10105-10115.

12. G. Bonneau, A. M. Peru, A. L. Flourat and F. Allais, *Green Chem.*, 2018, **20**, 2455-2458.
13. A. M. Peru, A. L. Flourat, C. Gunawan, W. D. Raverty, M. Jevric, B. W. Greatex and F. Allais, *Molecules*, 2016, **21**, 98801-98810.
14. S. H. Krishna, D. J. McClelland, Q. A. Rashka, J. A. Dumesic and G. W. Huber, *Green Chem.*, 2017, **19**, 1278-1285.
15. S. Kudo, N. Goto, J. Sperry, K. Norinaga and J. Hayashi, *ACS sust. Chem. Eng.*, 2017, **5**, 1132-1140.
16. M. M. Zanardi, A. G. Suarez and A. M. Sarotti, *J. Org. Chem.*, 2017, **82**, 1873-1879.
17. K. P. Stockton and B. W. Greatex, *Org. Biomol. Chem.*, 2016, **14**, 7520-7528.
18. F. Allais, A. L. Flourat, A. M. Peru, A. R. S. Teixeira, F. Brunissen and H. E. Spinnler, WO 2015165957 A1 20151105, 2015.
19. V. K. Brel, A. V. Samet, L. D. Konyushkin, A. I. Stash, V. K. Belsky and V. V. Semenov, *Mendeleev Commun.*, 2015, **25**, 44-46.
20. A. L. Flourat, A. A. M. Peru, A. R. S. Teixeira, F. Brunissen and F. Allais, *Green Chem.*, 2015, **17**, 404-412.
21. G. L. Larson and J. L. Fry, *Org. Reac.*, 2008, **71**, 1-737.
22. S. J. Craythorne, K. Anderson, F. Lorenzini, C. McCausland, E. F. Smith, P. Licence, A. C. Marr and P. C. Marr, *Chem. Eur. J*, 2009, **15**, 7090-7100.
23. T. Kawai, M. Isobe and S. C. Peters, *Aust. J. Chem.*, 1995, **48**, 115-131.
24. G. A. Tolstikov, M. E. Alder, I. N. Gaisina, F. A. Valeev and M. S. Miftakhov, *Zh. Org. Khim.*, 1993, **29**, 417-418.
25. M. Jaishankar, T. Tseten, N. Anbalagan, B. B. Mathew and K. N. Beeregowda, *Interdiscip Toxicol.*, 2014, **7**, 60-72.
26. https://rarediseases.org/rare-diseases/heavy-metal-poisoning/,
27. https://www.ema.europa.eu/documents/scientific-guideline/guideline-specification-limits-residues-metal-catalysts-metal-reagents_en.pdf,
28. A. C. Patterson-Orazem, B. Sullivan and J. D. Stewart, *Bioorg. Med. Chem.*, 2014, **22**, 5628-5632.
29. Y. A. Pompeu, B. Sullivan, A. Z. Walton and J. D. Stewart, *Adv. Synth. Catal.*, 2012, **354**, 1949-1960.
30. F. Shafizadeh, R. Furneaux, D. Pang and T. T. Stevenson, *Carbohydr. Res.*, 1982, **100**, 303-313.
31. F. Shafizadeh, R. H. Furneaux and T. T. Stevenson, *Carbohydr. Res.*, 1979, **71**, 169-191.
32. S. H. Krishna, T. W. Walker, J. A. Dumesic and G. W. Huber, *ChemSusChem*, 2016, **10**, 129-138.
33. I. Souchon, V. Athès, F.-X. Pierre and M. Marin, *Desalination*, 2004, **163**, 39-46.
34. G. Burgé, F. Chemarin, M. Moussa, C. Saulou-Bérion, F. Allais, H.-E. Spinnler and V. Athès, *J. Chem. Technol. Biotechnol.*, 2016, **91**, 2705-2712.
35. X. Wang, T. Saba, H. H. P. Yiu, R. F. Howe, J. A. Anderson and J. Shi, *Chem*, 2017, **2**, 621-654.