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In situ aldehyde generation for aldol addition reactions catalyzed by D-fructose-6-phosphate aldolase

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ABSTRACT

In situ coupling of aldehyde generation, by a mild alcohol oxidation, with an enzymatic aldol addition reaction, mediated by D-fructose-6-phosphate aldolase (FSA) has been investigated as an approach to improve the performance of the process. Four sustainable oxidation methods compatible with the activity and stability of the enzymatic aldol addition have been assayed. Among them, the laccase/O_2/2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) and alcohol oxidase gave the best results for the *N*-Cbz-aminoethanol to *N*-Cbz-glycinal (53%) and furfuryl alcohol to furfural (89%), respectively, followed by the aldol addition with hydroxyacetone catalyzed by FSA A129S mutant.

1. Introduction

The aldol addition reaction is one of the most powerful methods of forming carbon–carbon bonds [1]. Biocatalysis by means of aldolases offers a unique stereoselective and green tool to perform this transformation [2,3].

The aldol addition reaction involves an acceptor aldehyde that must be stable throughout the reaction as well as during its preparation, purification and manipulation conditions. Aldehydes are strong electrophiles that can undergo a number of side reactions with other nucleophiles present in the medium, including self-polymerization reactions and oxidation to acids. Moreover, they can deactivate or inhibit the aldolase either by non-selective binding or by irreversible Schiff base formation with the amino group of the catalytic lysine residue in Class I aldolases. To overcome this problem an *in situ* aldehyde generation by the corresponding alcohol oxidation through a sequential reaction under conditions compatible with the enzymatic aldol addition would be ideal (Fig. 1). The combination of both concepts, biocatalysis and multistep sequential, will have an economic and environmental benefit in the chemical production.

Oxidation is a central reaction in organic chemistry [4]. Several oxidizing reagents including permanganate and dichromate have been traditionally employed to accomplish this transformation. However, many methodologies use reaction conditions that are incompatible with the enzymatic activity and stability. Recently, much attention has been focused on the development of (bio)/catalytic methods [5] employing oxygen or hydrogen peroxide as highly attractive oxidants in terms of minimum energy use and waste generation. In this sense, tremendous advances in transition metal catalysis [6–9], and organocatalysis [10,11] applied to oxidation reactions have been achieved.

Moreover, biocatalysis is emerging as an additional pillar for environmentally benign oxidation catalysis in water [12,13]. Thus, biocatalytic approaches such as laccase/O_2/mediator system (LMS), alcohol oxidase (AO) and chloroperoxidase (CPO) are *a priori* good candidates.

Examples for the oxidase/aldolase coupling reactions are described in the literature [14–16]. In 1994 Fessner et al. developed an approach for the preparation of complex carbohydrates by enzymatic aldolization in which both the aldol donor and the acceptor components were generated *in situ* by air oxidation using microbial oxidases [14]. More recently, Siebum et al. briefly discussed the oxidation of 4-pentenol by alcohol oxidase (AO) followed by the condensation reaction catalyzed by 2-deoxyribose-5-phosphate aldolase (DERA) [16]. Similarly Wong and co-workers integrated the galactose oxidase (GO) catalyzed oxidation of glycerol to L-glyceraldehyde coupled with an aldolase reaction to produce L-fructose [15]. Their main drawback is that the oxidases usually lack of a broad substrate selectivity, therefore limiting their synthetic applications. Moreover, in some instances the need of metal cofactors used by the oxidases and class II aldolases such as L-rhamnulose-1-phosphate (RhuA), may be incompatible. Thus, alternatives must be provided to make the oxidation and aldol addition compatible to *in situ* transformations.

In this work, we endeavored to study the coupling of an *in situ* aldehyde generation by a green oxidation methodology with an enzymatic aldol addition reaction. We proposed to investigate

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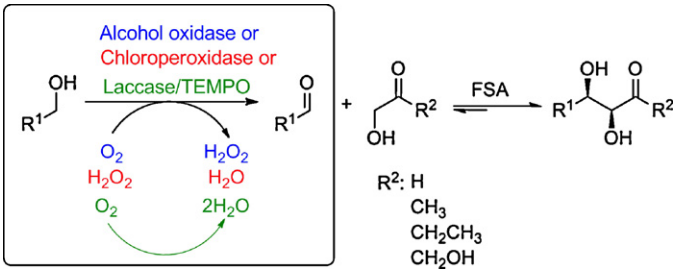

Fig. 1. Alcohol oxidation/aldol addition reaction cascade strategy.

four suitable catalysts for the alcohol oxidation, that were initially assumed compatible with the activity and stability of the enzymatic aldol addition step and they would only require the modification of the pH. Moreover, we avoided to use oxidoreductases using nicotinamide cofactors. Instead, our choices were systems that utilize oxygen or simple hydrogen peroxide, which makes them attractive and competitive for preparative organic chemistry.

First, the system laccase/O_2/2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) as mediator was selected. This has the advantage that TEMPO, which is regenerated by the laccase, is the oxidant agent and therefore the methodology is not restricted by the substrate selectivity of the enzyme [17–19]. TEMPO is already known to efficiently catalyze the oxidation of alcohols to carbonyl products [20,21]. Second, the alcohol oxidase (AO), a flavin-dependent alcohol oxidizing enzyme in the presence of O_2 will be tested as well [22]. AO oxidizes a range of (aliphatic) primary alcohols to the corresponding aldehyde with the concomitant production of H_2O_2 [23]. Under these conditions, catalase must be added to eliminate the hydrogen peroxide formed during the reaction, which is known to deactivate the enzymes. Third, chloroperoxidase (CPO) from *Caldaromyces fumago* can catalyze the oxidation of primary alcohols to aldehydes by the use of H_2O_2 as oxidant [24–26]. Peroxidases are generally restricted to oxidation of phenols to quinones *via* free radicals intermediates, but CPO is the exception to the general rule [25,27]. Fourth, a solvent free oxidation catalyst based on Au/Pd-TiO_2 [9] in the presence of O_2 or H_2O_2 was also tested. In terms of green chemistry, heterogeneous catalysis can enormously contribute to the oxidation reaction performance in a sustainable way. Solvent free oxidation reaction with heterogeneous catalyst could be also a good strategy for the multi-step reaction.

We propose class I D-fructose-6-phosphate aldolase (FSA) from *Escherichia coli* as catalyst for the aldol addition step. FSA has the advantage of accepting unphosphorylated didhydroxyacetone (DHA) analogues as the donor simplifying the process since there is no need to handle the phosphoester moiety [28,29].

2. Experimental

2.1. Materials

Furfuryl alcohol, *N*-Cbz-aminoethanol, hydroxyacetone, hydrogen peroxide (30%), palladium(II) choride, gold(III) chloride trihydrate, laccase from *Trametes versicolor* EC (1.10.3.2), alcohol oxidase from *Pichia pastoris* EC (1.1.3.13), galactose oxidase from *Dactylium dendroides* EC (1.1.3.9), and catalase from bovine liver were purchased from Sigma–Aldrich, CPO from *Saccharomices fumago* was purchased from Codexis, TEMPO was purchased from Evonik. Pd/Au-TiO_2 [9], FSA A129S and FSA A129S/A165G were obtained according to the literature [30].

2.2. HPLC analyses

HPLC analyses were performed with a XBridgeTM C18, 5 mm, 4.6 mm × 250 mm column from Waters (Massachusetts, USA). The solvent system used was solvent A (H_2O in the presence of 0.1% (v/v) trifluoroacetic acid (TFA)) and solvent B (MeCN/H_2O 4:1 in the presence of 0.095% (v/v) TFA), gradient elution from 10 to 100% B in 30 min, flow rate 1 mL/min, detection 215 nm. The amount of product was quantified from the peak areas using an external standard methodology.

2.3. Absorption of laccase onto Celite®

Enzyme (50 mg, 650 U) was dissolved in acetate buffer (1 mL, 50 mM, pH 4.5). To this solution Celite® (1 g) was added, and after mixing thoroughly the solid was dried under vacuum overnight. The lyophilized powder was directly use for the oxidation reaction.

2.4. Preparation of Pd/Au-TiO_2

Pd/Au-TiO_2 was prepared following the procedure described in the literature [9]. An aqueous solution of $HAuCl_4{\cdot}3H_2O$ (5 mL, 100 mg, 0.25 mmol, 0.05 M) and an aqueous solution of $PdCl_2$ (1 mL, 90 mg, 0.5 mmol, 0.5 M) were simultaneously added to TiO_2 (1.9 g). The paste formed was grown and dried at 80 °C for 16 h and calcined in static air at 400 °C. A characterization has been done, including TEM, elemental analysis, and BET surface area. The metal nanoparticles (2.5 nm average) were identified by transmission electron microscopy (TEM) technique. The final metal content was determined by inductive coupled plasma (ICP) and the amount of Pd and Au was according with the predicted. The Pd/Au-TiO_2 catalyst has a BET area of 54.4 m^2/g, which is in agreement with the area reported by Hutchings et al. [9].

2.5. One-pot two-step furfuryl alcohol oxidation by laccase/O_2/TEMPO and aldol addition catalyzed by FSA

2.5.1. Oxidation reaction

Reactions were conducted in 15 mL glass pressure vials. Furfuryl alcohol (69 μL, 0.8 mmol, 160 mM) and TEMPO (11.7 mg, 0.075 mmol, 15 mM) were dissolved in acetate buffer (5 mL, 50 mM, pH 4.5). This solution was aerated during 5 min. Then, laccase (4 mg, 50 U) or (24 mg, 325 U) depending on the experiment was added. The reaction was stirred in the presence of O_2 (2 atm) at 25 °C. Samples (50 μL) were withdrawn at 1, 3, 5, 7, and 24 h, diluted with MeOH (950 μL) and subsequently analyzed by HPLC.

2.5.2. Aldol addition reaction

To the reaction mixture resulting from the oxidation reaction (367 μL, 0.05 mmol, 100 mM final aldehyde concentration) was adjusted until pH 7 with Na_2CO_3. To this solution, HA (133 μL of 563 mM solution, 0.075 mmol; final HA concentration 150 mM) and FSA A129S (2.4 mg lyophilized powder, 1.9 mg protein, 18 U) were added. Reactions (500 μL total volume) were performed using 1 mL eppendorf tubes stirred with a vortex mixer (800 rpm) at 25 °C. Samples (50 μL) were withdrawn at 1, 3, 5 and 7 h, diluted with MeOH (950 μL) and subsequently analyzed by HPLC. The aldol addition product was identified by HPLC comparing with an authentic sample characterized in a scale up reaction.

2.5.2.1. Scale up of the aldol addition of HA to furfural by FSA A129S. General procedure: Reactions were conducted in 250 mL erlenmeyer flasks with 20 mL total reaction volume using the following reactant and enzyme concentration: Furfural (164 μL, 2 mmol, 100 mM final concentration), HA (204 μL, 3 mmol, 150 mM final concentration) and FSA A129S (72 mg lyophilized powder, 57.6 mg protein,

541 U) in triethanolamine (TEA) 500 mM pH 7.4 buffer. After 3 h reaction 50 μL sample was diluted with MeOH (950 μL) and the reaction was stopped by adding 20 mL of MeOH. Then, the MeOH was evaporated under vacuum and the precipitated enzyme filtered off. The filtrate was purified by column chromatography on Amberlite™ XAD™ 16 (Rohm and has) was packed into a glass column (45 cm × 1.5 cm). The Amberlite™ XAD™ 16 was equilibrated initially with H_2O and then the crude material (*i.e.* filtrate) was dissolved in water (50–70 mL) and loaded onto the column. Impurities were washed away with H_2O (2–3 column volumes). The fractions were analyzed by HPLC. Pure fractions were pooled and lyophilized obtaining the corresponding aldol adduct **(4)** (192 mg, 1.1 mmol, 58% isolated yield). The product was analized by NMR and compared with the one reported in the literature [31]. ^{1}H NMR (400 MHz, D_2O) δ = 7.54 (d, J = 0.8, Hz, 1H), 6.48 (dd, J = 4, 10 Hz, 1H), 6.46 (d, 7 Hz, 1H), 5.21 (d, J = 3.7 Hz, 1H), 4.66 (d, J = 3.7 Hz, 1H), 3.96 (m, 1H), 3.46 (m, 1H), 2.30 (s, 3H). ^{13}C NMR (100 MHz, D_2O) δ = 212.3, 153.8, 142.9, 110.5, 107.9, 78.7, 67.8 and 26.1

2.6. One-pot two-step furfuryl alcohol oxidation by alcohol oxidase and aldol addition catalyzed by FSA

2.6.1. Oxidation reaction

Reactions were conducted in 15 mL glass pressure vials. Furfuryl alcohol (86 μL, 200 mM) was dissolved in acetate buffer (5 mL, 50 mM, pH 7.5). This solution was aerated during 5 min. Then, catalase (2 mg, 7618 U) and alcohol oxidase (21 μL, 30 U) were added. The reaction was stirred in the presence of O_2 (2 atm) at 25 °C. Samples (50 μL) were withdrawn at 1, 3, 5, 7, and 24 h, diluted with MeOH (950 μL) and subsequently analyzed by HPLC.

2.6.2. Aldol addition reaction

To the reaction mixture resulting from the oxidation reaction (278 μL, 0.05 mmol, 100 mM final aldehyde concentration) was added HA (150 μL of a 500 mM solution), acetate buffer (72 μL, 50 mM, pH 7.5) and FSA A129S (2.4 mg lyophilized powder, 1.9 mg protein, 18 U). Reactions 500 μL (total volume) were performed using 1 mL eppendorf tubes stirred with a vortex mixer at 25 °C Samples (50 μL) were withdrawn at 1, 3, 5, and 7 h, diluted with MeOH (950 μL) and subsequently analyzed by HPLC.

2.7. Furfuryl alcohol oxidation reaction by CPO

Furfuryl alcohol (2.15 μL, 0.025 mmol, 25 mM) and H_2O_2 (2.8 μL, 0.025 mmol, 25 mM) were dissolved in acetate buffer (1 mL, 50 mM, pH 4.5). To this solution CPO (10 μL, 187 U) was added. The reaction was stirred in the presence of O_2 (2 atm) at 25 °C. Samples (50 μL) were withdrawn at 1, 3, 5, 7 and 24 h, diluted with MeOH (950 μL) and subsequently analyzed by HPLC.

2.8. Furfuryl alcohol oxidation reaction by Au/Pd-TiO_2

Reactions were conducted in 15 mL glass pressure vials. Furfuryl alcohol (1.1 g, 11.5 mmol) was aerated during 5 min. Then Au/Pd-TiO_2 (20 mg) was added. The reaction was stirred in the presence of O_2 (2 atm) at 25 °C. Samples (50 μL) were withdrawn at 1, 3, 5, 7 and 24 h, diluted with MeOH (950 μL), and subsequently analyzed by HPLC.

2.9. N-Cbz-aminoethanol oxidation by laccase/O_2/TEMPO system

Reactions were conducted in 15 mL glass pressure vials. *N*-Cbz-aminoethanol (10 mg, 0.05 mmol, 25 mM) and TEMPO (2.5 mg, 0.016 mmol, 8 mM) were dissolved in acetate buffer (2 mL, 50 mM, pH 4.5), acetate buffer (1 mL, 50 mM, pH 4.5)/CH_2Cl_2 (1 mL), acetate buffer (1 mL, 50 mM, pH 4.5)/hexane (1 mL) or acetate buffer (1 mL, 50 mM, pH 4.5)/AcOEt (1 mL). This solution was aerated during 5 min. Then, laccase (4 mg, 50 U) was added. The reaction was stirred in the presence of O_2 (2 atm) at 25 °C. Samples (50 μL) were withdrawn at 1, 3, 5, 7, and 24 h, diluted with MeOH (950 μL) and subsequently analyzed by HPLC.

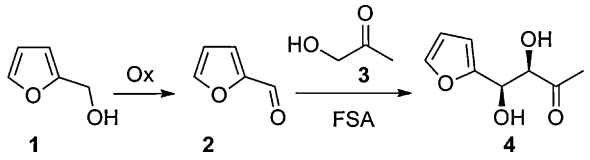

Fig. 2. Cascade reaction furfuryl alcohol to furfuraldehyde and subsequent aldol addition reaction with hydroxyacetone.

2.10. One-pot two-step N-Cbz-aminoethanol oxidation by laccase/O_2/TEMPO and aldol addition catalyzed by FSA

2.10.1. Oxidation reaction

Reactions were conducted in 50 mL open vials. *N*-Cbz-aminoethanol (234 mg, 1.2 mmol, 80 mM), TEMPO (70 mg, 0.45 mmol, 30 mM) or (140 mg, 0.9 mmol, 60 mM) depending on the experiment and hexadecyltrimethylammonium bromide (40 mg, 0.11 mmol, 7.3 mM) were dissolved in acetate buffer (10 mL, 50 mM, pH 4.5) and toluene (5 mL). Then, laccase (12 mg, 150 U) or (75 mg, 975 U) was added. The reaction was continuously aerated by a steel filter stone system and stirred at 25 °C. Toluene (1 mL) was added to the reaction every 30 min to replace the amount evaporated by the aereation. Samples (50 μL) were withdrawn at 1, 3, 5, 7, and 24 h, diluted with MeOH (950 μL) and subsequently analyzed by HPLC.

2.10.2. Aldol addition reaction

The previous reaction mixture containing the aldehyde (430 μL, 0.022 mmol, 45 mM final aldehyde concentration) hydroxyacetone (HA) (70 μL of 500 mM solution, 0.035 mmol; 70 mM final HA concentration) and FSA (2.4 mg lyophilized powder, 0.96 mg protein, 2.4 U) were added. Reactions 500 μL (total volume) were performed using 1 mL eppendorf tubes stirred with a vortex mixer at 25 °C Samples (50 μL) were withdrawn at 1, 3, 5, and 7 h, diluted with MeOH (950 μL) and subsequently analyzed by HPLC.

The aldol adduct was identified by HPLC comparing with an authentic sample characterized in a previous work [29].

3. Results and discussion

3.1. Selection of the oxidation methodology

Preparation of furan derivatives by carbon chain extension through aldol addition is of great interest for the transformation of biomass-derived molecules into added value compounds [31–33]. The consecutive oxidation/aldol addition reaction of furfuryl alcohol to furfuraldehyde and subsequent aldol addition reaction with hydroxyacetone catalyzed by FSA was first tested as a model process (Fig. 2). HA as well as the furfuryl alcohol contain primary alcohol functions prone to be oxidized, consequently a catalytic oxidation competition between both compounds can be anticipated. Thus, a one-pot cascade reaction method where all the reagents are added at the starting point could not be operated. Instead of that, the method of choice was a one-pot multi-step consecutive reaction in which the reagents are added at various points.

First, the oxidation step using the four selected methods was separately evaluated. The oxidation with laccase/O_2/TEMPO system reached high yields (Table 1, entries 1 and 2). The reactions were performed at room temperature, buffered at pH 4.5 under O_2

Table 1
Furfuryl alcohol oxidation with different systems.

Entry	Oxidation system (reaction time, h)	Units	Yield [%]
1	Laccase/TEMPO (7)[a]	325	89
2	Laccase/TEMPO (24)[a]	50	83
3	AO[b]	30	89
4	CPO[c]	187	47
5	Au/Pd-TiO_2[d]	–	0

[a] *Reaction condition*: 160 mM furfuryl alcohol, 15 mM TEMPO, 5 mL acetate buffer 50 mM, pH 4.5, 25 °C.
[b] *Reaction condition*: 200 mM furfuryl alcohol, 2 mg catalase, 5 mL acetate buffer 50 mM, pH 7.5, 25 °C, 24 h.
[c] *Reaction condition*: 25 mM furfuryl alcohol, 25 mM H_2O_2, 1 mL acetate buffer 50 mM, pH 4.5, 25 °C, 5 h.
[d] *Reaction condition*: 1 mL furfuryl alcohol, 20 mg Au/Pd-TiO_2, 25 °C, 24 h.

(2 atm). As summarized in Table 1, similar results can be obtained with approximately three-fold less amount of laccase, but with longer reaction times. Since the aldol additions are carried out at pH 7, a modification of the reaction pH was necessary for the aldol addition step when using this oxidation system.

Alcohol oxidase rendered also good conversions to furfuraldehyde. The oxidation was carried out in a slightly basic aqueous solution under oxygen atmosphere and catalase was added to decompose the H_2O_2 formed during the reaction avoiding deactivation of the enzymes. One of the main advantages of this oxidation system is that the pH of the oxidation step is the same as that of the aldol addition, simplifying the procedure.

Furfuryl alcohol oxidation reaction was also performed with chloroperoxidase (CPO) from *C. fumago* [26]. The reaction was carried out under mild conditions with H_2O_2 as oxidizing agent and aqueous buffer as solvent. Unfortunately, less than 50% conversion to aldehyde was achieved after 7 h. This result may be due to the enzyme's deactivation by the H_2O_2. CPO instability towards H_2O_2 and poor operational stability has already been reported in the literature [34].

The solvent free oxidation reaction catalyzed by Au/Pd-TiO_2 was not effective [9] in the oxidation of furfuryl alcohol.

3.2. Oxidation/condensation one-pot two-step consecutive reactions

The results obtained thus far revealed that laccase/O_2/TEMPO system and alcohol oxidase could be good candidates for the cascade aldehyde generation/aldol addition reaction. Taken into account our previous experience on FSA we choose the mutant A129S [35] to carry out the aldol addition reaction of hydroxyacetone to the *in situ* generated furfuraldehyde.

The aldol reaction was first coupled with the oxidation performed by laccase/O_2/TEMPO system. Both enzymes have different optimum operational pH, for that reason the pH was readjusted from 4.5 until 7 with sodium carbonate just before the enzymatic aldol addition. The reaction was performed at room temperature, and under these conditions, 50% conversion to the corresponding aldol adduct was reached after 5 h.

A more straightforward process was obtained when the oxidation was catalyzed by the alcohol oxidase. In this case, after the oxidation, the aldol addition was started just adding HA and the FSA A129S. After 6 h of reaction 80% of conversion to aldol adduct was achieved.

3.3. Synthesis of 1,4,5-trideoxy-1,4-imino-D-arabinitol (5-DDAB) by a chemo-enzymatic one-pot two-step consecutive reactions

Our ongoing project on the chemo-enzymatic synthesis of iminocyclitols has led us to apply the concept of oxidation/aldol addition to their synthesis. The key step in the strategy developed was the aldol addition reaction of dihydroxyacetone or analogues to *N*-carbobenzyloxy-aminoaldehydes (*N*-Cbz-aminoaldehydes) catalyzed by FSA [29]. Therefore, we first studied the oxidation of *N*-Cbz-2-aminoethanol itself being the precursor to the *N*-Cbz-glycinal (**6**). Aldol addition of hydroxyacetone to **6** leads to the aldol adduct (**7**), precursor of 1,4,5-trideoxy-1,4-imino-D-arabinitol (5-DDAB) (**8**) (Fig. 3) [36,37].

Considering the results obtained thus far for the furfuryl alcohol oxidation we selected the most successful methodologies to prepare the *N*-Cbz-glycinal (**6**): laccase/O_2/TEMPO and alcohol oxidase approaches. In a preliminary screening of both strategies, we found that laccase/O_2/TEMPO system gave some aldehyde formation whereas alcohol oxidase was not able to accept the *N*-Cbz-2-aminoethanol as substrate.

As starting point for the oxidation of *N*-Cbz-2-aminoethanol by laccase/O_2/TEMPO the reaction was performed in acetate buffer at pH 4.5. Under these conditions (Table 2, entry 1) a large amount of undesired carboxylic acid derivate was obtained which arises from the over-oxidation of the aldehyde formed during the reaction and its amount rises on increasing the reaction time and the conversion of the alcohol.

The oxidation of alcohols to aldehydes catalyzed by TEMPO/bleach-systems using bromide as cocatalyst, proceeds commonly in dichloromethaneas organic media [38]. Since the oxidation agent was in both cases the TEMPO, we hypothesize that the organic media would prevent the over-oxidation and therefore an organic-buffer two phase system was envisaged. With this strategy it was intended to remove the aldehyde from the water phase preventing the acid formation. Dichloromethane, hexane and ethyl acetate were the solvents of choice, and the reactions were performed under the same conditions as in the aqueous phase (Table 2, entries 2–4). As expected, the two phase systems improved the aldehyde formation but still the over-oxidation increased with the conversion. Ethyl acetate gave the best results in terms of aldehyde produced (42%).

It is known that enzyme immobilization can overcome in some instances the deactivation caused by the organic solvent. We envisaged the immobilization of laccase by a straightforward absorption onto Celite, as reported previously for proteases, lipases and alcohol dehydrogenase [39,40]. A reaction with immobilized laccase was performed under the same reaction conditions as those for using the enzyme in solution (Table 2, entry 5). With Celite-laccase preparation no acid formation was observed, but after 24 h of reaction only 10% conversion to aldehyde was achieved, which not improved substantially after 48 h (14%). It is likely that most of the laccase activity was lost during the adsorption process or that the water

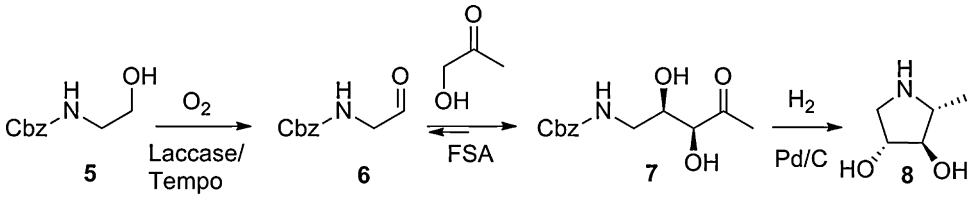

Fig. 3. 5-DDAB synthesis.

Table 2
N-Cbz-2-aminoethanol oxidation by the laccase/TEMPO system.

Entry	Reaction media	Conversion 5 h [%]	Aldehyde/acid 5 h [%]	Aldehyde 5 h [%]	Conversion 24 h [%]	Aldehyde/acid 24 h [%]	Aldehyde 24 h [%]
1	Buffer	21	55/45	12	76	19/81	14
2	CH_2Cl_2:buffer[a] (1:1)	4	100/0	4	12	95/5	11
3	Hexane:buffer[a] (1:1)	45	58/42	26	66	54/46	36
4	AcOEt:buffer[a] (1:1)	9	100/0	9	53	80/20	42
5	AcOEt:buffer[a] (1:1)[b]	–	–		10	100/0	10

Reaction conditions: 25 mM *N*-Cbz-aminoethanol, 8 mM TEMPO, 2 atm O_2, 2 mL acetate buffer 50 mM, pH 4.5.
[a] 1 mL acetate buffer 50 mM, pH 4.5: 1 mL solvent, 25 U laccase/mL.
[b] Laccase immobilized.

Table 3
N-Cbz-aminoethanol oxidation by continuous aeration and monophase system.

Entry	Oxidation conditions	Conversion 5 h [%]	Acid 5 h [%]	Conversion 24 h [%]	Acid 24 h [%]
1	30 mM TEMPO 10 U enzyme/mL	21	10	50	25
2	30 mM TEMPO 65 U enzyme/mL	37	10	91	38
3	60 mM TEMPO 65 U enzyme/mL	80	33	–	–

Reaction conditions: 80 mM *N*-Cbz-aminoethanol, O_2 bubbling, 10 mL toluene: 5 mL acetate buffer 50 mM, pH 4.5, 40 mg hexadecyltrimethylamonium bromide.

content (50%, v/v) was not optimal for the activity [39]. Therefore, the oxidation reaction was performed in ethyl acetate at $a_w = 0.753$ [41,42]. However, under these conditions no oxidation activity was observed. Other support materials such as polyamide (Accurel PA6 powder), which gave excellent activities with proteases were also tested for the laccase absorption but with similar results to those obtained with Celite [40].

It is reported in the literature [43] that when a two phase system was employed in the laccase/O_2/TEMPO system, the partition of the various components between the different phases may play a key role in the oxidation process. TEMPO, the alcohol and the aldehyde were mainly partitioned in the organic phase while laccase and the oxoammonium (*i.e.* oxidized TEMPO) are in the water phase. Laccase needs to react with TEMPO in order to produce the oxoammonium oxidant and it was assumed that this takes place at the water phase or at the organic-aqueous interphase. The oxidation of the alcohol by the oxoammonium cation takes place at the organic phase or at the organic-aqueous interphase too. Hence, it was hypothesized that the use of a system with large interfacial areas would probably benefit the laccase/O_2/TEMPO system. For that reason, we tested a ternary system, which consisted of toluene/buffer/hexadecyltrimethylamonium bromide (CTAB) (1:2:0.008). CTAB is a cationic surfactant widely used in micromicellar systems with application in enzyme catalyzed reactions [44].

Under these conditions, a significant improvement was achieved (Table 3). After 5 h reaction with 30 mM TEMPO and 65 units of laccase a 37% of conversion was reached with only 10% of acid (Table 3, entry 1). Leading the reaction run for 24 h, 91% of conversion with 62% of selectivity towards aldehyde formation was achieved (Table 3, entry 2). In this case, aeration was sufficient for the reaction to proceed and, since it caused solvent evaporation, 1 mL of toluene was added every 30 min.

On increasing the amount of TEMPO up to 60 mM, the conversion raised to 80% with 54% of aldehyde in 5 h (Table 3, entry 3), leading us to couple this reaction with the aldol addition reaction. The aldol addition of HA to *N*-Cbz-glycinal formed *in situ* was performed. First the pH of the reaction media was adjusted to 7 with sodium carbonate using FSA A129S/A165G as the optimum biocatalyst for this reaction [30]. The aldehyde was quantitatively converted to the adduct **7** after 3 h.

4. Conclusions

A synthetic approach based on *in situ* aldehyde generation by laccase/O_2/TEMPO or alcohol oxidase followed by an enzymatic aldol addition reaction mediated by FSA was assayed. Mild reaction conditions were employed making the system attractive from the environmental point of view. The concept was demonstrated by the oxidation of furfuryl alcohol to furfuraldehyde followed by the aldol addition of hydroxyacetone. This was further extended to the synthesis of a alpha-glucosidase inhibitor, (5-DDAB), by modifying the reaction conditions of the oxidation step. In aqueous media, treatment of *N*-Cbz-2-aminethanol with laccase/O_2/TEMPO system, lead to *N*-Cbz-glycine as major product. Organic-water two phase systems partially avoid the overoxidation, and in ethyl acetate-buffer two phase system the yield of aldehyde reached 42%. Further improvements were achieved by using an organic two-phase system in the presence of a surfactant. Thus in toluene/buffer/hexadecyltrimethylamonium bromide (CTAB) (1:2:0.008) the aldehyde formation was improved up to 53%.

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