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Short Communication

A robust chemo-enzymatic lactone synthesis using acyltransferase from *Mycobacterium smegmatis*

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ABSTRACT

The new application of acyltransferase, isolated from *Mycobacterium smegmatis* for the chemo-enzymatic Baeyer-Villiger oxidation of cyclic ketones to lactones was demonstrated. Acyltransferase exhibited high activity, and high stability under harsh reaction conditions, like oxidation with 60% aq. H_2O_2 at 45 °C. This paves the way to a novel robust chemo-enzymatic method for lactone synthesis with high yields.

1. Introduction

Baeyer-Villiger (BV) oxidation of cyclic ketones remains one of the most important protocols for the synthesis of lactones [1] with applications in the synthesis of antibiotics, steroids, pheromones and polymers [2].

Biocatalysis offers an environmentally friendly alternative for typical BV transformations which make use of organic percarboxylic acids [1]. The oxidation of ketones to lactones can be carried out using either Baeyer-Villiger monooxygenases (BVM) [3,4] or hydrolases such as *Candida antarctica* lipase B (CALB) [5–8] and esterases [9]. In the first case, using BVM, the enzyme catalyses the oxidation of ketones with oxygen and nicotinamide adenine dinucleotide phosphate NADPH as a source of electrons [3,4] to obtain lactones with enantioselectivities up to 100%. However, as BVMs are relatively expensive and poorly stable, the second option is preferred, in which the enzyme boosts the *in-situ* oxidation of long- or medium-chain carboxylic acids or ethyl acetate with hydrogen peroxide to generate peracids used to oxidise ketones to lactones in the second (chemical) step [5–8]. Clearly, this single-pot chemo-enzymatic approach is far more elegant and attractive commercially.

The search for enzymes that are active and stable in this reaction was crucial and the results were already reported in several papers. Hydrogen peroxide and the peracid generated during the reaction are inactivating reagents for enzymes. To improve the enzyme stability and to allow the effective recycling of enzymes the immobilization techniques can be used [10]. The most studied was Novozyme-435, *i.e.* CALB immobilized on acrylic resin [5]. But very efficient cross-linked enzyme aggregates (CLEAs) with perhydrolase, in a chemo-enzymatic reaction have been also proposed [7]. Our previous studies demonstrated very good activity of CALB immobilized on siliceous materials with organosilanes terminated with alkyl groups [6] or in ionic liquids [8].

The acyltransferase, more recently isolated from *Mycobacterium segmentis* (MsAcT), appeared to demonstrate in the presence of hydrogen peroxide a perhydrolysis: hydrolysis activity ratio 50-fold greater than the common lipases (CALB included) [11]. It was immobilized and used as a paint additive, catalyzing peracid formation even under those conditions [12,13]. Therefore, MsAcT emerges as a natural candidate to replace lipases also in the chemo-enzymatic Baeyer-Villiger oxidation of cyclic ketones to lactones.

In this light, we deemed it important to test the MsAcT performance in this reaction and to compare it with those shown by free CALB or immobilized as Novozyme-435. Needless to say that the reaction under study is of a major practical significance.

2. Experimental

2.1. Materials

30% and 60% aq. H_2O_2 and UHP were purchased from Acros Organics. Cyclic ketones and native CALB [5,000 LU/G] were purchased from

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Sigma Aldrich. Novozyme-35 was donated by Novozymes. Enzym MsAcT was produced and the activity determined as described earlier [14].

2.2. General method for Baeyer-Villiger oxidation

The ketone (0.25 mmol) and 0.5 ml of ethyl acetate (5.09 mmol) were introduced into a 25 ml round-bottom flask and the contents of the flask was shaken. Next, 4 mg of MsAcT was introduced, and 60% aq. H_2O_2 (0.50 mmol) was added dropwise. The flask was sealed with a septum and mixed in a thermostated shaker (±0.5 °C) with orbital stirring at 250 rpm at 35 °C for 2 h to 5 days, depending on the reaction rate. Periodically, 10 μl of the sample diluted with 0.7 ml of dichloromethane was collected during the reaction to monitor the progress of the reaction utilising GC (Perkin Elmer Clarus 500 chromatograph with SUPELCOWAX™ 10 column (30 m × 0.2 mm × 0.2 μm) with *n*-decane as an external standard.

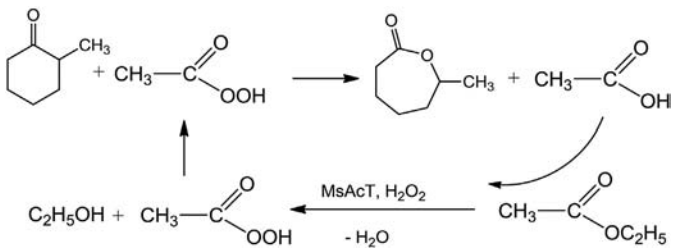
The structure of the products were confirmed using GC–MS analysis (Agilent Gas Chromatograph 7890C equipped with a HP-5 MS column (30 m × 0.25 mm × 0.25 μm; MS Agilent 5975C, EI ionization 70 eV, and the results were compared to NIST/EPA/NIH Mass Spectral Library.

3. Results and discussion

As can be seen from Scheme 1 the applied chemo-enzymatic method of lactone synthesis involves MsAcT as the biocatalyst of peracid formation, hydrogen peroxide as oxidant and ethyl acetate as both the peracid precursor and solvent. As a model ketone 2-methylcyclohexanone was used.

The experiments were performed under the reaction conditions recommended for this reaction (25 °C, molar ratio of ketone to 30% aq. H_2O_2 1: 2) [6]. At first, they aimed at discriminating the regions of specific kinetic control of the chemo-enzymatic Baeyer-Villiger reaction. They were made by varying the amount of MsAcT in the range of 2–7 mg, while keeping the amount of a model ketone constant (0.25 mmol; 2-methylcyclohexanone). For a fixed value of molar ratio of the ketone to 30% aq. H_2O_2 (1:2) and an excess of ethyl acetate the rate of lactone formation at 25 °C appeared to depend on the biocatalyst content, provided it was ≤4 mg (Fig. 1). Those findings delineated the region of effective control of ketone oxidation by the created peracid (4–7 mg of MsAcT). Since differences in the reaction courses carried out using 4 and 7 mg of the enzyme per 0.25 mmol of ketone appeared to be small, therefore all further studies were performed using 4 mg of the enzyme.

At this stage we also checked the influence of ethyl acetate and the possibility to replace its excess with a buffer. These studies showed that the 5.09 mmol of ethyl acetate (0.5 ml) per 0.25 mmol of ketone (approximately, only 5% of ethyl acetate is consumed for the reaction) is the most effective and that the addition of the buffer has insignificant influence on the reaction rate (Fig. 2). The latter phenomenon could be explained by an extensive hydrophobicity of MsAcT active center [11, 13–16]. In all cases, with the addition of buffer or not, the reaction system was always biphasic according to the use of water solution of H_2O_2 and the creation of water molecule as a by-product in the reaction.



Scheme 1. Model chemo-enzymatic Baeyer-Villiger oxidation studied in this work.

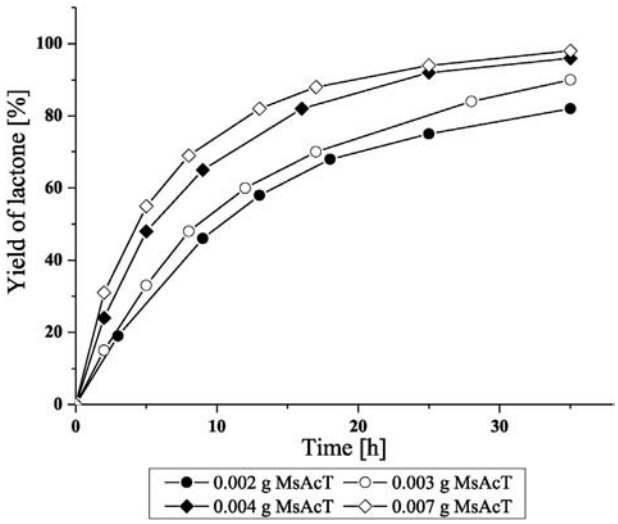


Fig. 1. Effect of MsAcT content on the BV oxidation of 2-methylcyclohexanone (0.25 mmol) with 30% aq. H_2O_2 (0.50 mmol) in ethyl acetate (5.09 mmol, 0.5 ml) at 25 °C.

The effect of the buffer was also checked for two forms of CALB lipase; a native (liquid) and its immobilized form - Novozyme 435. As can be seen from Fig. 3 the presence of water appeared to exert a strong negative effect on their activity, regardless the biocatalyst form. This could be ascribed to two factors: (i) specific structure of the lipase, especially the presence of hydrophobic polypeptide chain (lid or flat), isolating its active centre from the reaction medium [17], (ii) hydrolysis of ethyl acetate which lowered the pH and this brought the reaction to a halt.

Further studies aimed to determine the effect of oxidising agent (30 and 60% aq. H_2O_2, urea hydrogen peroxide UHP). They showed that the most reactive is 60% aq. H_2O_2 (Fig. 4). A twofold molar ratio of 60% aq. H_2O_2 to the ketone was large enough to ensure optimum kinetics, *i.e.* very similar in value to that obtained using a fourfold excess of 30% aq. H_2O_2. It is noteworthy, that the idea of using 60% aq. H_2O_2 was also aimed to probe the MsAcT performance under harsh reaction conditions, since the exposure of CALB to high concentration of aq. hydrogen peroxide resulted in its complete deactivation [18]. Anhydrous UHP appeared to be poorly reactive under these reaction conditions. The efforts for the isolation of enzyme after the reaction were unsuccessful. The use of enzyme immobilization methods may produce improvements in the enzyme performance, as was already described in the literature. [19,20].

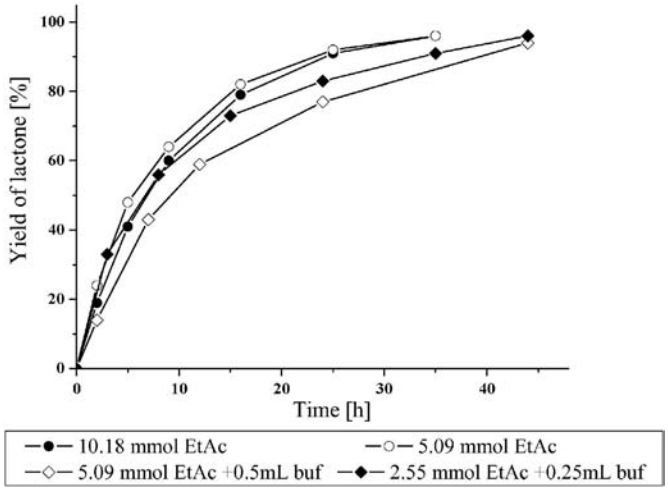


Fig. 2. The influence of the solvent on the BV oxidation of 2-methylcyclohexanone (0.25 mmol) with 30% aq. H_2O_2 (0.50 mmol) and MsAcT (0.004 g) at 25 °C.

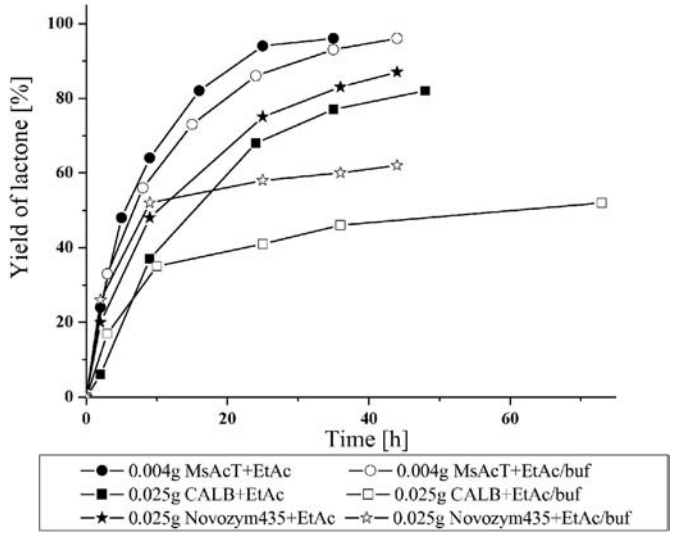


Fig. 3. Comparative study of MsAcT, Novozyme-435 and a native CALB (water solution) performance in the BV oxidation of 2-methylcyclohexanone (0.25 mmol) with 30% aq. H_2O_2 (0.50 mmol) in ethyl acetate (5.09 mmol, 0.5 ml) or buffer/ethyl acetate (0.25 ml/0.25 ml, 2.55 mmol) at 25 °C.

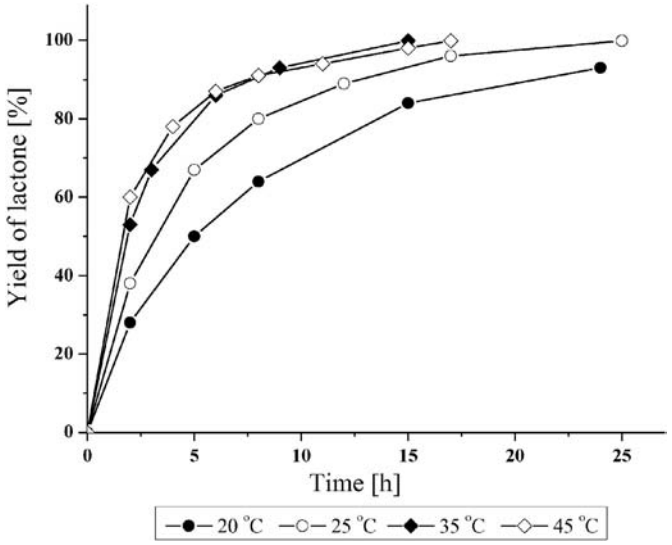


Fig. 5. The influence of the temperature on the BV oxidation of 2-methylcyclohexanone (0.25 mmol) with 60% aq. H_2O_2 (0.50 mmol) and MsAcT (0.004 g) in ethyl acetate (5.09 mmol, 0.5 ml).

More remarkably, the stability of MsAcT in the presence of 60% aq. H_2O_2 appeared to be high even at higher temperature (35-45 °C; Fig. 5). Control experiments in the absence of the enzyme using 30% and 60% of aq. hydrogen peroxide for the oxidation of 2-methylcyclohexanone at 35 °C demonstrated that the reactions do not occur without enzyme even in these harsh conditions.

Finally, the enzyme was examined in the synthesis of various lactones from the corresponding ketones to determine its practical potential. As can be seen from Table 1 cyclic ketones were readily oxidised to their corresponding lactones in high yields (84-99%) under the optimised conditions, in the presence of 60% aq. H_2O_2 at 35 °C.

Very reactive cyclobutanone, which is a strained ketone was oxidised to γ-butyrolactone in 2 h with 99% of yield. The oxidation of 2-adamantanone and norcamphor gave their corresponding lactones also in high yields, respectively after 4 and 7 h. The non-strained ketone cyclohexanone, known for being much more difficult to oxidise, gave 84% ε-caprolactone after 120 h. Substituents in 4 position in the cycle like methyl, ethyl, propyl and phenyl resulted in the prolongation of reaction times up to 120 h compared to the 2-methylcyclohexanone oxidation (11*h*). As expected all products of the oxidation of prochiral ketones and 2-methylcyclohexanone were racemic mixtures, since the enzyme only catalyses the peracid formation but not the Baeyer-Villiger reaction.

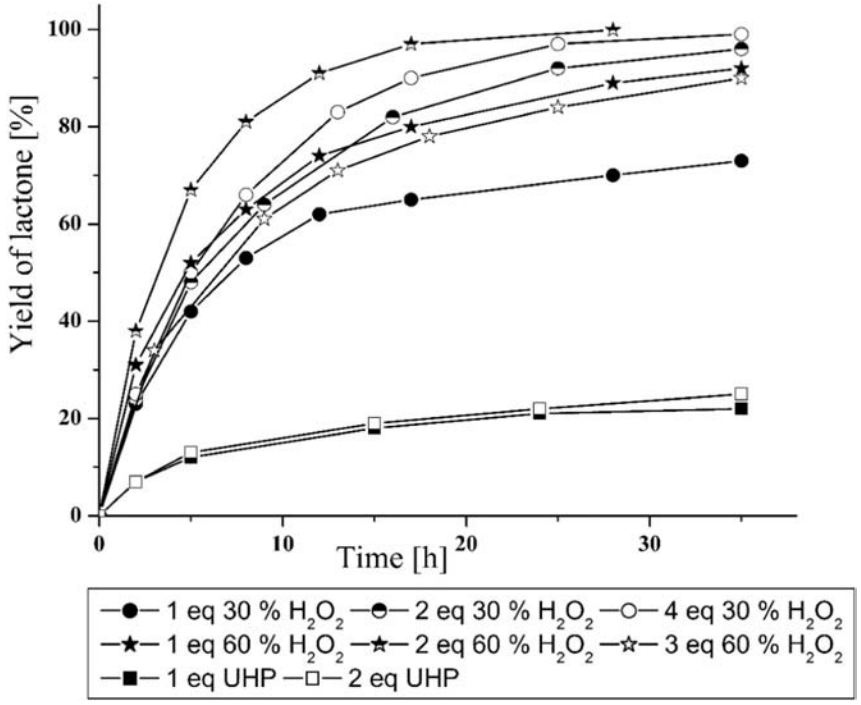
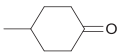
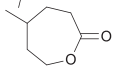
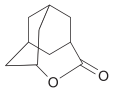
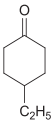
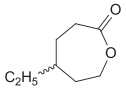
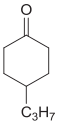
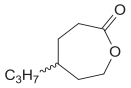
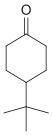
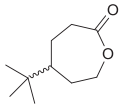
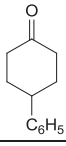
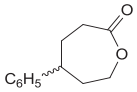


Fig. 4. The influence of the structure and amount of primary oxidant on the BV oxidation of 2-methylcyclohexanone (0.25 mmol) with MsAcT (0.004 g) in ethyl acetate (5.09 mmol, 0.5 ml) at 25 °C.

Table 1
Oxidation of selected cyclic ketones to lactones.[a]

Entry	Ketone	Lactone	Time [h]	Yield [%]
1			120	84
2			11	99
3			60	99
4			4	98
5			7	96
6			2	99
7			110	98
8			120	98
9			90	99
10			120	85

[a] Reaction conditions: cyclic ketone (0.25 mmol), MsAcT (0.004 g), ethyl acetate (5.09 mmol, 0.5 ml), 60% aq. H_2O_2 (0.5 mmol), 35 °C.

4. Conclusions

To summarise, the acyltransferase examined in this study appeared to be very effective mediator of the chemo-enzymatic Baeyer-Villiger reaction. It revealed some of the most important advantages: very high activity and good stability demonstrated in oxidation of various cyclic ketones to the corresponding lactones. The proposed environmentally benign chemo-enzymatic method avoids the synthesis of peracid in the separate process, which in the proposed method is generated *in situ* in the reaction system during the enzymatic stage. Currently, in our laboratory the method for the immobilization of MsAcT is studied.

Acknowledgments

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