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ORIGINAL PAPER

An Easy and Efficient Fluorescent Method for Detecting Aldehydes and Its Application in Biotransformation

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Abstract Water-soluble aldehydes (acetaldehyde, propionaldehyde) and non-water-soluble aldehydes (butyraldehyde and phenylacetaldehyde) were easily detected by an efficient fluorescent method with 5-aminofluorescein as probe. Under optimal detection conditions, 5-aminofluorescein could selectively respond to aldehydes with high sensitivity in comparison with other carbonyl compounds like ketones and acids. Thus, the proposed method was used to monitor microbial oxidation and succeeded in trapping transiently-produced aldehydes during biotransformation of primary alcohols by *Gluconobacter oxydans*.

Keywords Fluorescent probe · 5-Aminofluorescein · Aldehydes · Microbial oxidation

Introduction

Aldehydes are acknowledged as the important carbonyl compounds. Some short-chain volatile aldehydes are the key components of toxic substances in environment and industry [1], while many aliphatic aldehydes play an important role as indispensable components of many flavors in food [2]. Aldehydes can be naturally produced through plant emitting [3], chemical reactions [4, 5], and by mild biotransformations [6]. Particularly, biotransformation is a useful and complementary tool for preparative organic chemistry [7]. The microbial oxidation of primary alcohols to the carboxylic acids in mild water phase is a widespread transformation and undergoes two procedures induced by the action of alcohol dehydrogenases firstly and the performance of aldehyde dehydrogenases secondly [8]. The intermediate aldehydes are transiently-produced species from the first oxidation step without normal accumulation (Fig. 1a) so that the specific function of two kinds of dehydrogenases in such a microbial oxidation transformation cannot be figured out [9, 10]. Although aldehydes can be extracted into organic solvent in water-isooctane biphase system in avoid of further oxidation [11], this method cannot help us to understand the action of dehydrogenases in water phase in detail. Therefore, prolonging the life of transiently-produced aldehydes in water phase for the detection is of great importance for the study of biotransformation mechanism and the activities of alcohol dehydrogenase and aldehyde dehydrogenases.

Because aldehydes are volatile and activated, the demand for their stable products obtained through chemical derivatization is crucial for their detection. In view that hydrazine reagents are well-known groups of such derivatizing chemicals [12–15], they have been combined with different analytical methodologies, such as high-performance liquid chromatography (HPLC) [16–19] and gas chromatography (GC) [20], so as to detect the existence of aldehydes. However, these derivative reagents can react with both aldehydes and ketones at the same time so that

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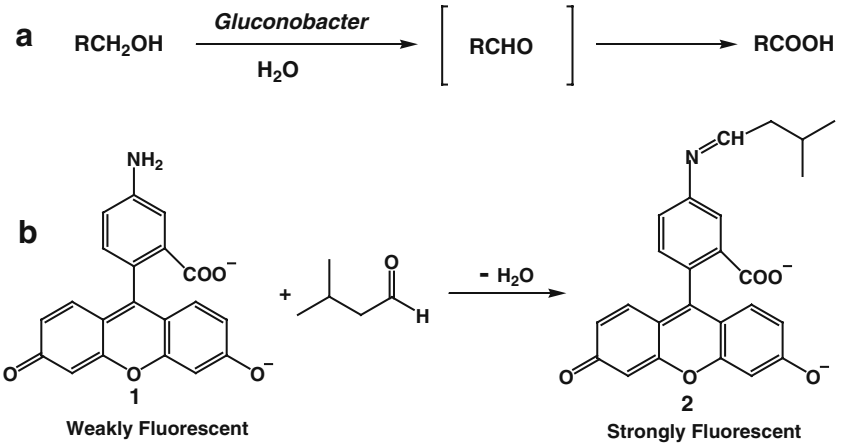

Fig. 1 **a** The microbial oxidation of primary alcohols. **b** Model reaction for the evaluation of the assay

they are not suitable for the application of selective detection of aldehydes in complicated samples. Moreover, these combined techniques are employed to detect the ordinary aldehyde chemicals rather than intermediate species. It is a pity that no papers were reported to successfully detect trace amount of aldehydes which are transiently-produced during enzyme oxidation of primary alcohols to acids.

In comparison with other reported methods, fluorescence detection has its apparent advantages over others, thus, a lot of fluorescence derivatization techniques have been developed for aldehydes [21, 22] and many new methods have been evolved in recent years [23, 24]. Such fluorescent probe method has also been widely accepted as a useful tool for detecting short life substances like free radicals [25, 26]. In this paper, we aimed to detect the transient-produced aldehydes by such a fluorescent probe method. By the derivation reaction between a derivative reagent 5-aminofluorescein (Fl-NH_2) and aldehydes, Fl-NH_2 had been proved to be an efficient fluorescent probe for trapping transiently-produced aldehydes during enzyme oxidation. This result exhibits two extraordinary aspects. On one hand, a trace of aldehydes with short life can be trapped by this probe showing its high sensitivity. On the other hand, this fluorescent probe only reacts with aldehydes (not ketones or acids), which indicates its high selectivity.

Experimental

Instrumentation

Fluorimetric analysis was performed with the Fluoroskan Ascent Microplate Fluorometer from Thermo-Labsystems (USA). The UV–visible absorption spectra of the probe were measured with a Cary-50 spectrophotometer (Varian, U.S.A.) in a 1 cm quartz cuvette. All pH measurements were made with a Sartorius Basic PH Meter PB-10 (Germany). IR Spectra was recorded on Nicolet iS10 FT-IR from Thermo-Labsystems (USA). Electrospray mass spectrometry (ESMS) was performed with a Quattro Micro tandem quadrupole mass spectrometer (Waters Corporation, Micromass Ltd., UK) in negative ion modes.

Chemicals

5-Aminofluorescein (Fl-NH_2) and isovaleraldehyde were purchased from Sigma-Aldrich. Aldehydes including acetaldehyde, propionaldehyde, butyraldehyde and phenylacetaldehyde, and all the substrates of different kinds of alcohols were purchased from Sinopharm. All other chemicals were obtained at the highest purity available from common commercial suppliers.

Synthesis and Characterization

In order to verify the reaction between Fl-NH_2 and isovaleraldehyde, the product should be synthesised and characterized. Fl-NH_2 (33.5 mg, 0.10 mmol) was dissolved in methanol (5 mL) in a round bottom flask, then isovaleraldehyde (22 μL, 0.20 mmol) and hydrochloric acid (36%, 139 μL) were added in turn. The reaction mixture was heated under reflux and stirred for 30 min. Then, more acetaldehyde (0.20 mmol) was added and the reaction was continued for 1 h. After removal of excess solvent under vacuum, the obtained solid powder was subjected to silica-gel gel chromatography (eluted with acetone: n-hexane = 1: 2) to afford the fluorescent product **2** as orange solid. H-NMR (600 MHz, DMSO, 25 °C, tetramethylsilane (TMS)): 11.17 (1H), 7.88−7.21 (6H), 4.91 (1H), 4.04 (2H), 3.70−3.62 (3H), 2.43 (2H), 1.23−1.14 (6H). IR (KBr, cm^{-1}): 1664.7 (C=N); LC-MS (API-ESI): **2**, [M]−, *m/z* Calcd. 416.2 found 416.2; Fluorescein, $[M]^-$, *m/z* Calcd. 332.3 found 331.2

Analytical Methods

In fluorescent assay, 150 μL of each reagent were added in the sequence of probe solution (Fl-NH_2, final concentration 0.10 mM), PBS solution (pH 7.4, final concentration 50 mM) and aldehyde samples (isovaleraldehyde, acetaldehyde, propionaldehyde, butyraldehyde and phenylacetaldehyde, final concentration 0.50 mM). In biotransformation, 150 μL of probe solution (Fl-NH_2, final concentration 0.10 mM), 50 μL of PBS solution (pH 7.4, final concentration 150 mM), 100 μL of water sample extracts were added sequently. Then the mixtures were diluted with ethanol to 1.5 mL. Fluorescence was determined on a microplate fluorometer with a 96-well plate (λex/λem=485 nm/538 nm). All measurements were performed in triplicate. For the standard curve detection, a 1.5 mM stock solution of fluorescent product **2** in ethanol: PBS solution (50 mM 7.4) (9/1, *v*/*v*) was prepared and then diluted to obtain solutions in the range of 0.10 μM to 10 μM.

Microorganisms and Culture Conditions

The strain (*Gluconobacter oxydans* NCIMB 621) was from official collection (NCIMB: National Collection of Industrial and Marine Bacteria, Aberdeen, UK). Submerged cultures were carried out in a GlyY medium (glycerol 25 g L^{-1}, yeast extract 10 g L^{-1}, pH 6.0) into 1 mL. The strain was incubated at 30 °C and resting cells were obtained by centrifugation (5,000 rpm at 4 °C, 5 min) of 24-h cultures and washing with PBS solution (pH 6.0, 50 mM). The OD600 value is 1.13 and all the related experiments were performed with the same cell concentration.

Biotransformations

Small-scale experiments were carried out with resting cells resuspended in PBS solution (pH 6.0, 50 mM). Substrates (8 g L^{-1}) were directly added to the cell suspensions and whole-cell bioconversions were conducted at 30 °C in an incubator shaker (200 r/min). In experiments, biotransformations of propanol and isopropanol were performed in PBS solution (pH 6.0, 50 mM). For other substrates including n-butanol, isoamyl alcohol and benzyl alcohol, water system (PBS solution, pH 6.0, 50 mM) and a two-liquid phase system of PBS solution and isooctane (1/1 *v*/*v*) were used. According to the studies of Molinari et al. [7, 27], the biotransformation time of various alcohols differed. Thus, biotransformations were done in a period of 60 min. During the process the first sample was extracted at 5 min, and others were extracted in the same intervals (15 min, 30 min, 45 min, and 60 min). After that, water samples of different substrates were extracted and 100 μL of each liquid sample was immediately used for fluorescent detection after filtration.

Results and Discussion

Model Reaction

In our work, isovaleraldehyde was selected as the model aldehyde in the analytical experiments, because isoamyl alcohol had been proved to be better substrate for some strains of *Gluconobacter oxydans* [27]. Our strategy is based on the intramolecular photo-induced electron transfer mechanism of the probe. Fl-NH_2 is weakly fluorescent because of its intramolecular self-quenching, while it can exhibit fluorescence properties of fluorescein when the amine's electron lone pair is made unavailable for electron transfer through covalent or electrostatic binding process [28] (Fig. 1b). After encountered with isovaleraldehyde, **2** showed strong yellow-green fluorescence, which were apparent color and fluorescence changes from the light yellow solution of Fl-NH_2.

Optimization of Experimental Variables

Optimal experimental variables were obtained by the model reaction of Fl-NH_2 and isovaleraldehyde.

Effect of pH Values

In the experiment, the pH of the medium was determined in the range of 6.5 to 8.0 using phosphate buffered saline (PBS) solution. As is shown in Fig. 2, under neutral and weakly alkaline conditions, Fl-NH_2 showed good response to isovaleraldehyde. Thus, a solution of pH 7.4 was employed

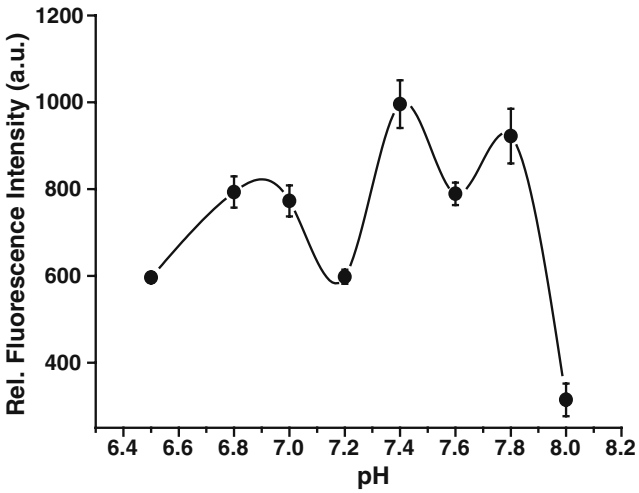

Fig. 2 Effect of pH values. The addition sequence of reagents: probe solution (0.10 mM), PBS solution (50 mM), isovaleraldehyde (0.50 mM)

throughout the analytical experiment in order to obtain the higher signal-to-noise (the following optimum experimental variables were also chosen according to this principle).

Effect of Probe Concentration

In our experiment, we also tested the effect of different probe concentrations with the reaction system of Fl-NH_2 and isovaleraldehyde. The result in Fig. 3 showed that Fl-NH_2 was best responded to isovaleraldehyde at the concentration of 0.10 mM. A higher probe concentration induced a decrease in fluorescent intensity mainly due to the aggregation [29–31] and self-absorption effect [32, 33] of probe molecules.

Effect of Isovaleraldehyde Concentration

The concentration of detected substance also has effect on the reaction system. Thus, we detected the effect of isovaleraldehyde concentration with the reaction between Fl-NH_2 and isovaleraldehyde. The result showed that Fl-NH_2 gradually responded to isovaleraldehyde as the concentration increased from 0.0030 μM to 1.0 mM, and 0.50 mM of isovaleraldehyde was chosen to be the optimum condition in the experiment (Fig. 4).

Kinetic Assay

The kinetic assay was performed in the experiment to get the optimum reaction period of Fl-NH_2 and isovaleraldehyde. As shown in Fig. 5, the reagent blank showed weak fluorescence emission and no significant intensity change was observed in a period of 60 min (line A). Upon the addition of isovaleraldehyde, fluorescence increase was immediately observed until 10 min and then fluorescence increased slightly in a period of 60 min (line B). Thus, a reaction time of 30 min was chosen throughout the analytical experiment.

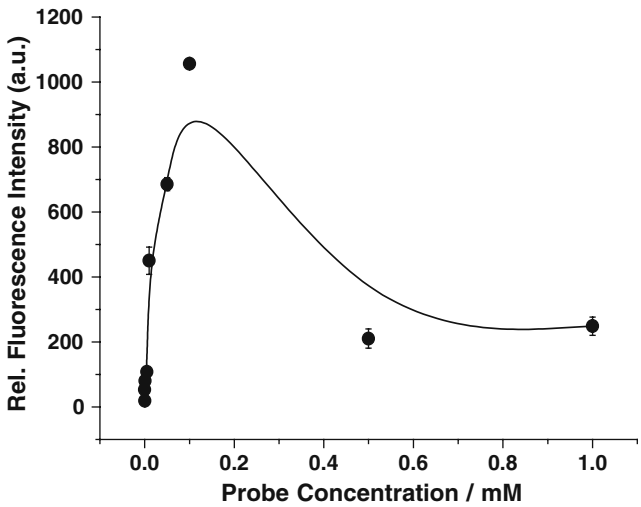

Fig. 3 Effect of probe concentration. (Isovaleraldehyde concentration: 0.50 mM; PBS solution: pH 7.4, 50 mM)

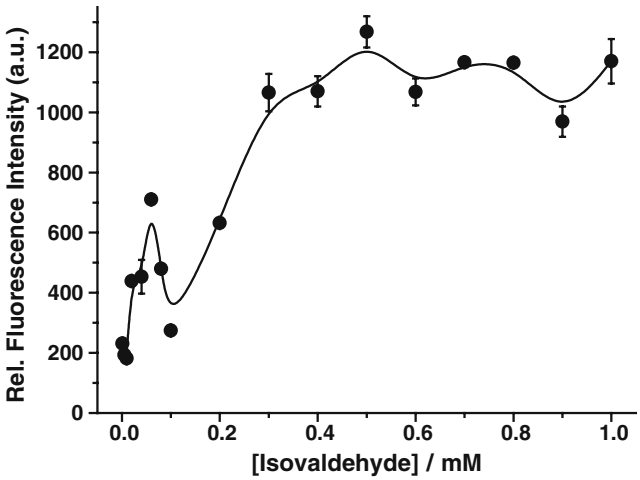

Fig. 4 Effect of isovaleraldehyde concentration. (Probe concentration: 0.10 mM; PBS solution: pH 7.4, 50 mM)

Standard Curve and Absorption Spectra

After synthesis of fluorescent product **2** from the reaction between Fl-NH_2 and isovaleraldehyde, the standard curve was determined to correlate fluorescence intensity with the concentration of **2** (Fig. 6a). A good linearity was detected in the range of 0.10 μM to 10.0 μM and the regression equation was $(F - F_0) = 90.69 + 2030.38 \times [\mathbf{2}]\,(\mu\mathrm{M})$ with a linear coefficient of 0.996. The limit of detection (LOD) of the

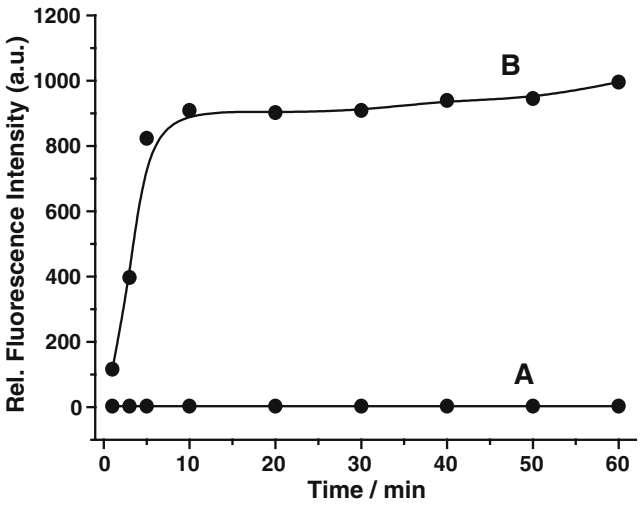

Fig. 5 Kinetic assay of Fl-NH_2 (0.10 mM) and its reaction with isovaleraldehyde (0.50 mM) in ethanol: PBS solution (pH 7.4, 50 mM) (9/1 *v*/*v*). Fluorescence responses were recorded on a microplate fluorometer (λex/λem=485/538 nm) with a 96-well plate

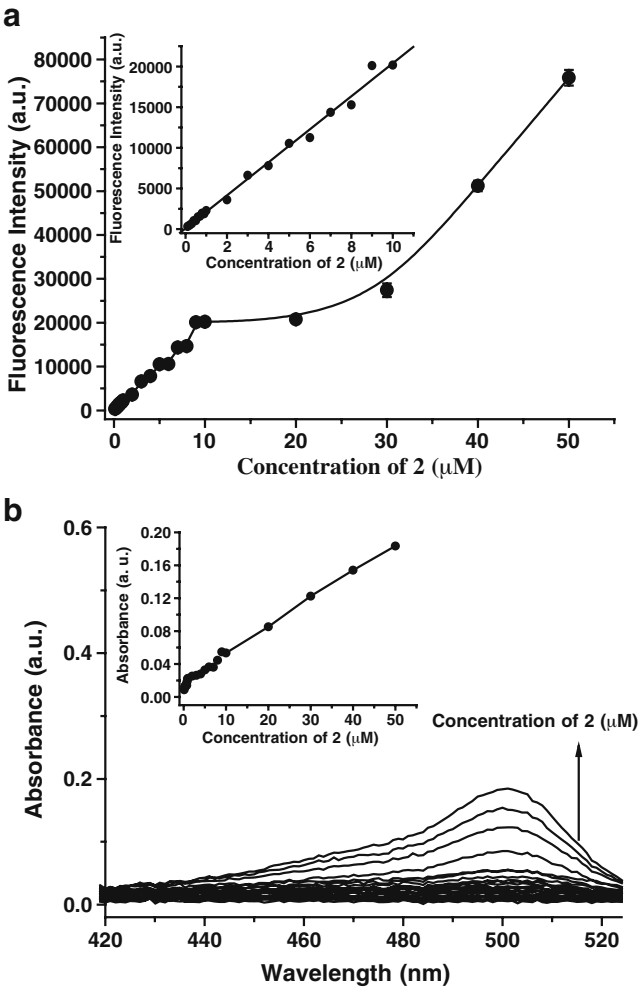

Fig. 6 **a** Carlibration curve for **2** determined in ethanol: PBS (pH 7.4, 50 mM) (9/1 *v*/*v*) with the concentration range of 0.10 μM to 50 μM. The insert figure is the linear range of the measurement. In the synthesis of **2**, acid was used as catalyst, while in real-time reaction of Fl-NH_2 and aldehydes, acid could affect the fluorescence of Fl-NH_2. Thus, relative low fluorescence intensities were observed in the detection of aldehydes (Figs. 2–5, 7 and 8) compared to that of the synthesized product **2** (Fig. 8). For the reaction of Fl-NH_2 and isovaleraldehyde, the Ka values between fluorescent intensity and concentration of acidic condition and mild condition were 2030.38 a. u. μM^{-1} and 9,103 a.u. mM^{-1}. This mainly due to the mild reaction system without an acidic catalyst and the volatilization of aldehydes during the detection. **b** Absorption spectra of **2** were detected in ethanol: PBS (pH 7.4, 50 mM) (9/1 *v*/*v*) with the concentration range of 0.10 μM to 50 μM

proposed method was 6.94 nM. Furthermore, the absorption spectra were recorded with the same concentration range of **2**. As shown in Fig. 6b, absorbance of **2** increased with its concentration varied from 0.10 μM to 50.0 μM. As a result, the molar extinction coefficient was calculated to be 129,100 L. $mol^{-1}.cm^{-1}$ by using Lambert—Beer's law These data clearly indicated that Fl-NH_2 could be used to detect isovaleraldehyde qualitatively and quantitatively.

Reactivities of Fl-NH_2

Many derivatizing reagents for aldehydes could react with other carbonyl compounds including ketones and acids [22, 34]. During the detection of aldehydes, it is necessary to assure the selectivity of the probe to aldehydes over other substances. As a result, the reactivities of Fl-NH_2 toward different alcohols and carboxyl compounds at a concentration of 0.50 mM were investigated by measuring their respective fluorescent responses. An error of ±5.0% in the relative fluorescence intensity was considered tolerable. As shown in Fig. 7, under the same detection conditions, Fl-NH_2 could respond obviously both to short-chain aliphatic aldehydes (C_2–C_5) and aromatic aldehyde (2-phenylacetaldehyde), while no fluorescent responses were detected for alcohols, ketone and acids. These results demonstrated that Fl-NH_2 could interact with aldehydes to make distinct change in fluorescence intensity, even in competition with alcohols and other carboxyl compounds.

Biotransformation of Primary Alcohols

Owing to its chemical and fluorescent properties, Fl-NH_2 should be suitable for detecting aldehydes in liquid mixture during microbial oxidation. In the work of Molinari et al. [7], aldehyde production through alcohol oxidation with *Gluconobacter oxydans* were studied. *Gluconobacter oxy-*

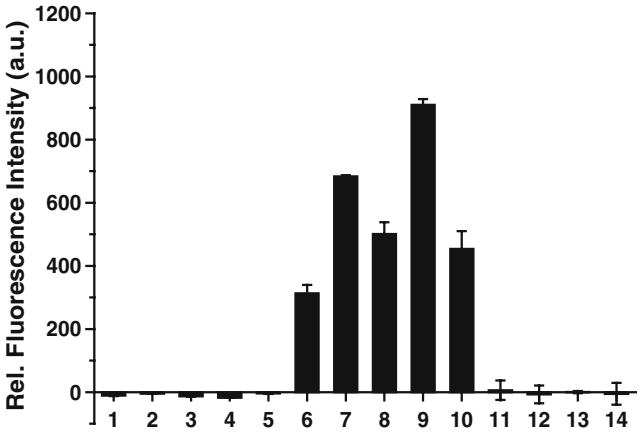

Fig. 7 Fluorescence responses of 0.10 mM Fl-NH_2 with different alcohols and carboxyl compounds (0.50 mM) in ethanol: PBS solution (pH 7.4, 50 mM) (9/1 *v*/*v*) for 30 min. The black bars represent: *1*: Propanol (Ka_1: −113.33 a.u. mM^{-1}), *2*: Isopropanol (Ka_2: −46.27 a. u. mM^{-1}), *3*: n-Butanol (Ka_3: −131.33 a.u. mM^{-1}), *4*: Isoamyl alcohol (Ka_4: −175.23 a.u. mM^{-1}), *5*: 2-Phenylethanol (Ka_5: −45 a.u. mM^{-1}), *6*: Acetaldehyde (Ka_6: 3,127 a.u. mM^{-1}), *7*: Propionaldehyde (Ka_7: 6,833 a.u. mM^{-1}), *8*: n-Butyraldehyde (Ka_8: 5,200 a.u. mM^{-1}), *9*: Isovaleraldehyde (Ka_9: 9,103 a.u. mM^{-1}), *10*: 2-Phenylacetaldehyde (Ka_{10}: 4706.67 a.u. mM^{-1}), *11*: Acetone (Ka_{11}: 20 a.u. mM^{-1}), *12*: Acetic acid (Ka_{12}: −75 a.u. mM^{-1}), *13*: Propionic acid (Ka_{13}: 2 a. u. mM^{-1}), 14: Butyric acid (Ka_{14}: −53 a.u. mM^{-1})

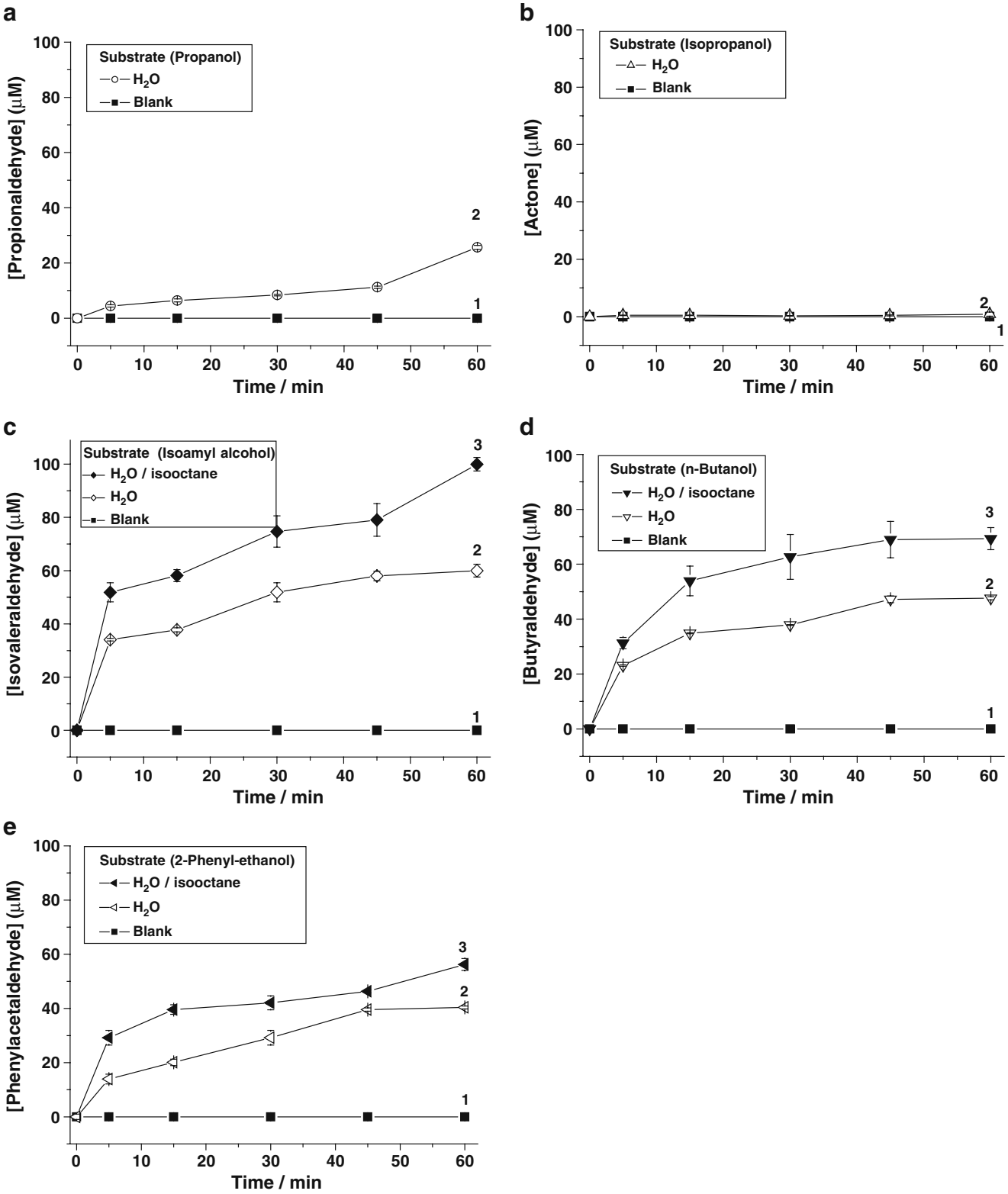

Fig. 8 Detection of transiently-produced aldehydes during microbial oxidation of alcohols with *Gluconobacter oxydans* NCIMB 621 in 60 min. Figure **a** represents the biotransformation of water-soluble aldehyde propanol in water phase. Figure **b** represents the biotransformation of isopropanol in water phase. Figure **c**, **d** and **e** represent the biotransformations of isoamyl alcohol, n-butanol and 2-phenylethanol in two kinds of liquid systems (water and water/isooctane) respectively. Fluorescent responses were recorded on a microplate fluorometer as in chemical experiments

dans NCIMB 621, which was maintained in acidic condition, was proved to be able to convert primary alcohols into respective acids in mild liquid phase and could be used for the production of aldehydes in the water-isooctane biphase system. Thus, the biotransformations of different substrates were performed separately and catalysed by resting cells of *Gluconobacter oxydans* NCIMB 621 in our experiment. To test the feasibility of Fl-NH_2 in detecting transiently-produced aldehydes, various alcohols were used as substrates including propanol, isopropanol, n-butanol, isoamyl alcohol, and 2-phenylethanol (see "Experimental" Section).

These alcohols can easily dissolve in water, but their oxidation products have different solubilities: propionaldehyde and acetone are water-soluable, while n-butyraldehyde, isovaleraldehyde and 2-phenylacetaldehyde only dissolve in organic solvents. As the use of water-isooctane biphase system could accumulate non-water-soluble aldehydes into isooctane phase, the transformations of alcohols were carried out in two conditions: propanol and isopropanol were converted in water system, while n-butanol, isoamyl alcohol, and 2-phenylethanol were converted in both water system and a water-isooctane biphase system(1/1 *v*/*v*) as comparison. During biotransformations, aldehydes were continuously produced, therefore, microliter of water samples of these mixtures were extracted at intervals and used for fluorescence detection.

For the biotransformation of different alcohols, the changes in fluorescent intensity were detected. As Ka value is available from Fig. 7, the final reaction products of different aldehydes could be quantitatived. As the results showed, blanks of bacteria liquid of *Gluconobacter oxydans* NCIMB 621 had no fluorescent responses (Fig. 8a–e, line 1). In water system, the transformation of propanol to propionaldehyde was obvious in a period of 60 min (Fig. 8a, line 2), while the product acetone from isopropanol was not detected, which was in accordance with the results of reactivity assay experiment (Fig. 8b, line 2).

For water sample extracts of microbial oxidation of isoamyl alcohol, n-butanol and 2-phenylethanol, products responses were detected upon reaction with Fl-NH_2 (Fig. 8c–e). Isoamyl alcohol showed a higher conversion rate in the biphase systems (Fig. 8c, line 3), in comparision to water phase transformation (Fig. 8c, line 2), mainly due to the effect of accumulation of liposoluble aldehydes in isooctane [11]. The same results were observed for n-butanol and 2-phenylethanol (Fig. 8d and e). Besides, under optimal biotransformation conditions for isoamyl alcohol, it exhibited the highest conversion rate to isoveraldehyde (Fig. 8c, line 2 and line 3), compared with the conversion of n-butanol and 2-phenylethanol to n-butyraldehyde and 2-phenylacetaldehyde respectively (Fig. 8d, 10 E, line 2 and line 3). These results clearly showed that Fl-NH_2 could trap both water-soluble and non-water-soluble aldehydes in transiently-produced forms during microbial oxidation of primary alcohols.

Conclusion

In general, 5-aminofluorescein was used for the detection of some kinds of aldehydes. After its application in microbial oxidation of primary alcohols, the feasibility of the proposed method was demonstrated to be an easy and efficient method for trapping transiently-produced aldehydes. The study was expected to be useful to develop fluorescent indicators for aldehydes, and to understand the function and mechanisms of dehydrogenases. Work along this line is still in progress in our laboratory.

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