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High throughput automated colorimetric method for the screening of L-lactic acid producing microorganisms

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ABSTRACT

Lactic acid is a valuable and fully degradable organic acid with promising applications in poly-lactic acid production (Taskila S and Ojamo, 2013 [1]). Despite their efficiency, the cost of the current lactic acid bio-processes is still an obstacle to this application (Miller et al., 2011 [2]). To ameliorate lactic acid producing strains, researchers are using mutations and metabolic engineering techniques, as well as medium optimization. All these studies necessitate a good and high throughput screening method. Currently, researchers mostly use HPLC methods which often necessitate sample preparation, are not stereospecific and do not allow high throughput. To help optimizing L-lactic acid production, we developed a high throughput colorimetric method inspired by the blood L-lactic acid detection method used for diagnosis (Lin et al., 1999 [3]).

- Two sequential enzymatic reactions using L-lactate oxidase, peroxidase and ABTS (2,2′-azino-di-[3-ethylbenzthiazoine-sulfonate]), a chromogenic peroxidase substrate, are used to quantify L-lactate between 13.8 and 90 mg/l.
- The accuracy of the method was ascertained before automation.
- The method was successfully applied for the direct determination of L-lactate content in fungal culture supernatants.

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Method details

Lactic acid is the building block for Poly-Lactic Acid (PLA), a biodegradable polymer which could replace petroleum-based plastics. With the environmental urge for ecofriendly products, the demand for this biomaterial increases the demand for lactic acid, and the market is expected to reach 1 million tons by 2020 [1]. Lactic acid bacteria are well known to secrete large amounts of L and/or D-lactic acid as a product of carbohydrate metabolism [1]. However, production costs of lactic acid using LAB are still too high restricting PLA use as a convenient product [2]. Overall production costs can be decreased by improving production step using (i) more efficient, either natural or genetically modified, microorganisms, (ii) optimizing culture

Abbreviations: ABTS, 2,2′-azino-di-[3-ethylbenzthiazoine-sulfonate]; HRP, horseradish peroxidase; LLOD, lower limit of detection; LLOQ, lower limit of quantification; LOD, L-lactate oxidase; LA, L-lactate concentration.

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conditions, e.g. low-nutrient medium with low-cost carbon source, low pH etc. For this type of studies, a fast and high throughput screening method for lactic acid production would be useful.

The colorimetric detection of L-lactate uses two sequential oxido-reduction reactions. The first one is the conversion of L-lactate and O_2 into pyruvate and H_2O_2, catalyzed by the stereospecific L-lactate oxidase (LOD). In the second reaction, catalyzed by the horseradish peroxidase (HRP), the chromogenic substrate ABTS (2,2′-azino-di-[3-ethylbenzthiazoine-sulfonate]) is oxidized by the H_2O_2 produced in first reaction. The oxidized ABTS absorbs at 420 nm and dyes the reaction mixture in green. The ABTS oxidation is proportional to L-lactate concentration which can be calculated from a standard curve of known L-lactate concentration. The method was adapted from L-lactic acid detection methods used in diagnosis [3]. For that purpose, the reaction parameters: optimal enzyme/substrate ratios, incubation time, detection and quantification lower limits were determined. The automated assay was then performed by measuring the accuracy and the repeatability of the results obtained using a pipetting robot. Finally, to prove the practicality and accuracy of the microplate method, the assay was tested with fungal culture supernatants and the method was compared with HPLC using a method previously used for organic acid determination [4]. This two-steps quantification method can be performed at room temperature, directly on diluted supernatants. Considering measurements in duplicates with one calibration curve per plate, 80 samples could be analyzed in 1 h and the robot could potentially run analysis 24 h per day (i.e. 1920 samples per day). As a comparison, more than 3 days would be necessary to run 80 samples with a classical HPLC method.

Preparation of the reaction mixture

The stereospecific LOD (50 U/ml, SORACHIM SA) and HRP (150 U/ml, Sigma–Aldrich) stock solutions were prepared from powders diluted in phosphate reagent buffer 0.1 M, pH 6. ABTS (5 mM, Sigma–Aldrich) and L-lactate (5 g/l, Sigma–Aldrich) stock solutions were prepared by dissolving the reagent powder in deionized water. These stock solutions were stored at 4 °C before use.

Determination of the enzyme ratios and incubation time

The first step was to determine the best ratio between the two enzymes and the end point time of the reactions. In manual assays, the HRP final concentration was fixed to 1.5 U/ml while three LOD final concentrations were assayed: 0.25, 1.5 and 2.5 U/ml. The L-lactate standards, 0, 5, 10, 20, 40, 60, 80, and 100 mg/l, were prepared with proper dilutions of the stock solution. The reaction mixture (final volume of 200 μl) contained 20 μl of 1 M phosphate reagent buffer pH 6, 20 μl of ABTS (5 mM), 2 μl of HRP (150 U/ml), 10, 60 or 120 μl LOD (50 U/ml), 10 μl L-lactate standard solutions and deionized water. Microplates were 10 s slowly stirred and incubated at 37 °C for 0, 5, 10, 20, 30 or 40 min in a ThermoScientific Multiscan GO microplate reader. For each incubation time, microplates were 10 s slowly stirred before measuring the absorbance at 420 nm. The zero absorbance was set on air. Regardless the L-lactate concentration, the end point of the reaction was reach after 5 min for mixture containing 1.5 or 2.5 U/ml of LOD, while 20 min were required with 0.25 U/ml of LOD (Fig. 1A).

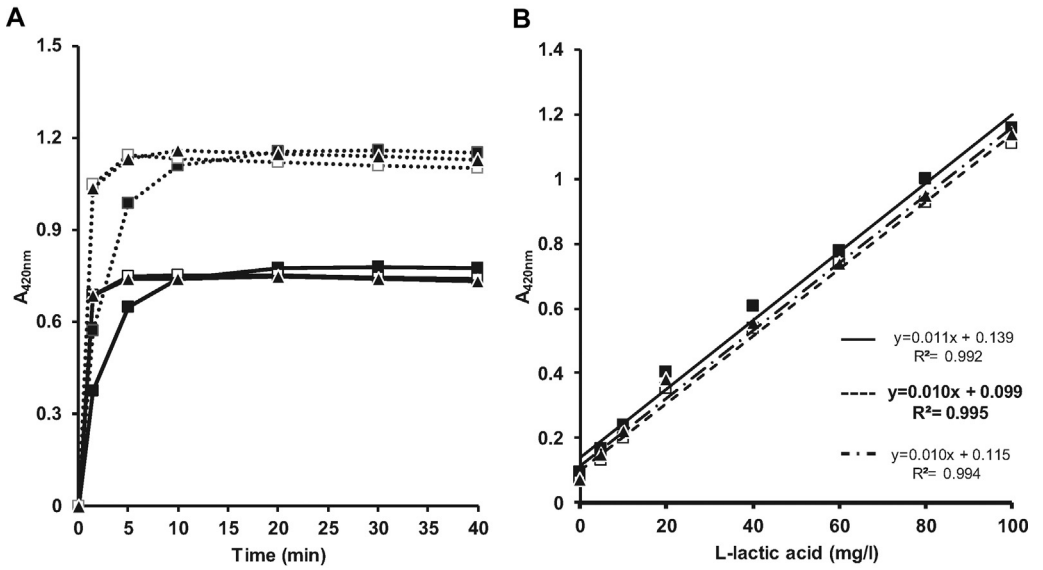

Fig. 1. Kinetics and linear regression for L-lactate quantification by colorimetric microplate method. (A) Kinetics of the reaction obtained with mixes containing 0.25 (■), 1.5 (□) or 2.5 (▲) U/ml of LOD; at two standard L-lactate concentrations: [LA] = 60 mg/l (—) and 100 mg/l (– – –). (B) Standard curves obtained after 30 min incubation with mixes containing 0.25 (■), 1.5 (□) or 2.5 (▲) U/ml of LOD. The linear regressions and equations obtained for each LOD concentration are shown in the figure: 0.25 (—), 1.5 (– – –) or 2.5 (–·–) U/ml of LOD.