



A high-throughput screening method based on the Mizoroki-Heck reaction for isolating itaconic acid-producing fungi from soils



Mei Sano^a, Hikari Kuroda^a, Hitomi Ohara^a, Hiroshi Ando^b, Keiji Matsumoto^b, Yuji Aso^{a,*}

^a Department of Biobased Materials Science, Kyoto Institute of Technology, 1 Hashigami-cho, Matsugasaki, Sakyo-ku, Kyoto, 606-8585, Japan

^b Corporate R&B Planning Department, Kaneka Corporation, 2-3-18 Nakanoshima, Kita-ku, Osaka, 530-8288, Japan

ARTICLE INFO

Keywords:

Microbiology
High-throughput screening
Itaconic acid
Aspergillus terreus
Iodine test
Mizoroki-Heck reaction

ABSTRACT

In this study, we report a novel method based on the Mizoroki-Heck reaction followed by an iodine test for the screening of itaconic acid-producing fungi from soils. This method is simple, rapid, and requires 10 μL of culture; results are obtained within 1.5 h. The detection limit of itaconic acid in the cultures was 0.13 mM. This is the first report on the direct screening of itaconic acid-producing fungi using a coupling reaction.

1. Introduction

Itaconic acid (IA), produced by fungi, is a versatile vinyl compound functioning as a building block for synthetic polymers and exerts enzyme-inhibitory and anti-inflammatory effects [1, 2, 3]. Owing to its properties, the demand for IA is increasing [4]. To date, fungal IA producers such as *Aspergillus terreus* [5, 6], *A. itaconicus* [7], *Ustilago maydis* [8], *Candida* sp. [9], and *Pseudozyma antarctica* [10] have been isolated from soil, plants, and fermented foods. Typically, researchers have isolated organic acid producers on agar plates with a pH indicator and subsequently identified them as IA producers based on comprehensive analyses of their metabolites [10]. However, the isolation of many consequential IA producers is limited due to the lack of suitable methods to screen IA producers directly.

To date, several studies have reported various approaches to quantify IA in microbial cultures. HPLC with a UV detector is a typical method for IA quantification [11]. Kreyenschulte *et al.* [12] used ^1H NMR as a detector of HPLC for IA detection. Recently, a biosensor consisting of an IA-inducible promoter and LysR-type transcriptional regulator in engineered bacteria was used for the quantification of IA intra-/extracellularly [13]. However, these methods require special devices and microbes. Other than these methods, the bromine absorption method for IA detection is simple [14]; however, it shows low specificity due to the presence of other compounds having internal C–C double bonds, which also get brominated.

The Mizoroki–Heck coupling reaction is a specific coupling reaction

of terminal C–C double bonds with aryl halide [15, 16]. We previously quantified acrylic acid in aqueous solutions containing contaminants, by labeling with iodobenzene (IB) using the coupling reaction [17]. These results suggested that IA in microbial cultures containing contaminants could be labeled in the same way. Iodide anion formed by the reaction and subsequently oxidized with NaNO_2 under acidic conditions can be quickly detected by an iodine test with starch, since the concentration of iodide ion is proportional to the reaction transition [18, 19]. In this study, a high-throughput screening method was developed for isolating fungi producing IA by using the Mizoroki-Heck reaction followed by an iodine test (Fig. 1).

2. Materials and methods

2.1. Labeling reaction and iodine test

The condition of labeling reaction was optimized according to the method reported by Sano *et al.* [17]. For the labeling reaction, 1–10 mM IA in water (10 μL), 180 mM IB in DMSO (10 μL), 375 mM K_2CO_3 in water (4 μL), and 4.5 mM $\text{Pd}(\text{OAc})_2$ in DMSO (2 μL), were added to a 96-well PCR plate (volume 0.1 ml) and heated at 80 $^\circ\text{C}$ for 1 h. Concentrated HCl (1.75 μL) was added to the mixture to adjust the pH of the mixture to 1. After the labeling reaction, 10 μL of the mixture was injected and monitored using HPLC (LaChrom Elite, Hitachi High-Technologies, Japan), equipped with a COSMOSIL 5C18-AR-II column (Nacalai Tesque, Kyoto, Japan). The reactants were eluted using

* Corresponding author.

E-mail address: aso@kit.ac.jp (Y. Aso).

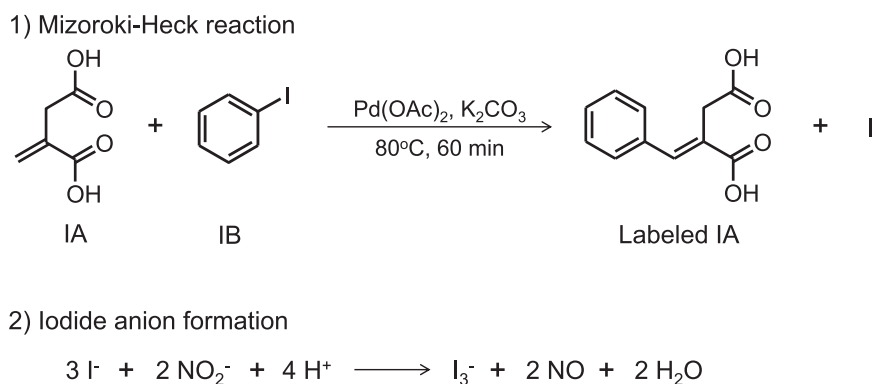


Fig. 1. The Mizoroki-Heck reaction of itaconic acid (IA) with iodobenzene (IB), followed by the iodine test.

water/acetonitrile/trifluoro acetic acid solutions. The flow rate was 1 mL/min, and the eluate was monitored at an absorbance of 210 nm. The column was calibrated using chemically synthesized 2-benzylidene succinic acid solutions as a standard.

The reaction transition was monitored using the iodine test in a 96-well microplate. After the labeling reaction, 50 μL each of 5% soluble starch and 5% NaNO_2 were added to the reaction mixture and then cooled at -20°C for 10 min. The developed color was measured with a microplate reader (iMark, Bio-Rad, USA) at 595 nm.

2.2. Isolation of fungi producing IA from soils

To evaluate the developed screening method, we isolated fungal samples from soils. Nineteen soil samples were collected from various places in Japan and plated onto potato dextrose agar supplemented with 25 $\mu\text{g}/\text{mL}$ chloramphenicol. After 7 days of incubation at 30°C , 240 filamentous colonies were randomly selected and then cultivated using 700 μL of the GM broth (per 1 L: 20 g glycerol, 0.154 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.19 mg $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 0.46 g NH_4NO_3 , 15.4 mg KH_2PO_4 , 96 mg CaCl_2 , 1.2 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.3 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) [20], in a 96-deep-well plate kept on a shaker at 1600 rpm for 7 days at 30°C (DWMAx M-BR-034P, TAI-TEC, Japan). After cultivation, 10 μL from each culture was used for labeling through the Mizoroki-Heck reaction.

The isolated strain was identified based on DNA sequence analysis of the ribosomal RNA gene [21].

2.3. LC-MS analysis

LC-MS was carried out using a Prominence UFLC-XR (Shimadzu, Kyoto, Japan) equipped with a Cadenza CD-C18 column (Imtakt, Kyoto, Japan) and an ESI-MS detector microTOF II-kp (Bruker, Massachusetts, United States). After the labeling reaction, 10 μL of the mixture was injected and monitored. The reactants were eluted using 0.1% formic acid/acetonitrile solutions with a flow rate of 0.2 mL/min. The absorbance of the eluate was monitored at 210 nm, and mass analysis was done in a negative ion mode.

3. Results and discussion

The labeled IA was detected at 12.5 min on the HPLC chromatogram when IB was added (Fig. 2A and B). The concentration of IA detected showed a good correlation ($R^2 = 0.9219$) with the IA applied, in the concentration range of 0–10 mM (Fig. 2C), indicating that a specific labeling reaction occurred in a dose-dependent manner. The reaction yield after labeling was calculated as 0.485 mol/mol based on the slope of the formula ($y = 0.485x$), suggesting that the labeling reaction was affected by steric hindrance provided by the carboxyl group at the C-2 position of IA. The concentration of detected IA was 0.502 mM when 1 mM IA was subjected to the labeling reaction (Fig. 2A and B). The reaction yield (0.502 mol/mol) was in good agreement with the calculated reaction yield.

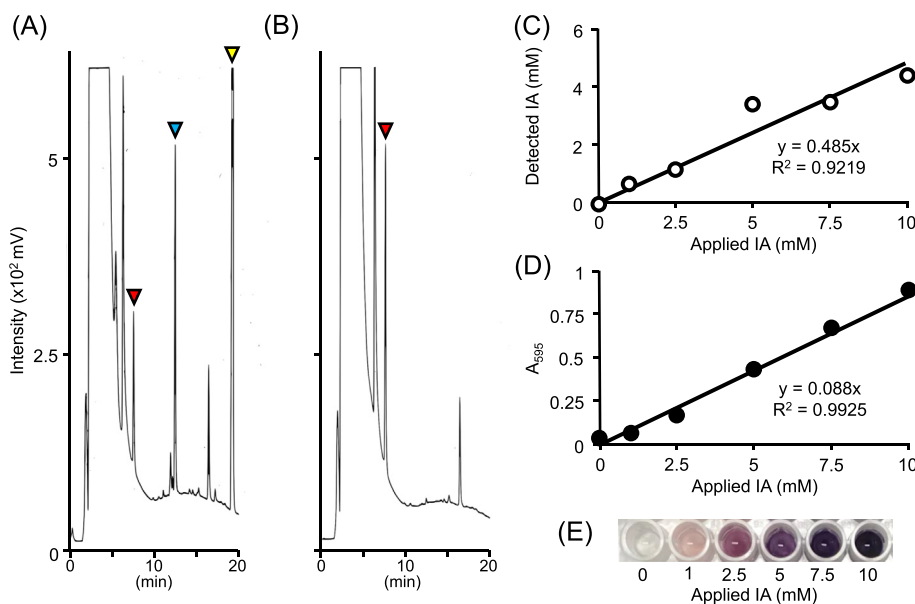


Fig. 2. Characterization of itaconic acid (IA) after labeling reaction with iodobenzene (IB). HPLC analyses of labeled IA after labeling reaction with 1 mM IA in the presence (A) or absence (B) of IB. Red, blue, and yellow triangles indicate unreacted IA, labeled IA, and unreacted IB, respectively. (C) Plots prepared from the concentrations of IA used in the labeling reactions vs. the concentrations of the labeled IA quantified by HPLC analyses. The labeling reactions were performed with IA over 0.1–10 mM at 80°C for 60 min. The data represent the average of the measurement of three independent experiments. Note: error bars are obscured by the symbols. (D) Plots prepared from the concentrations of IA used in the labeling reactions vs. the absorbances of color developed by the iodine test. After the labeling reaction, 50 μL each of 5% soluble starch and 5% NaNO_2 were added to the reaction mixtures in a 96-well microplate and cooled at -20°C for 10 min. The color developed was measured with a microplate reader at 595 nm. The data represent the average of the measurements of three independent experiments. Note: error bars are obscured by the symbols. (E) A microplate containing the reaction mixtures with 0–10 mM IA after adding soluble starch and NaNO_2 .

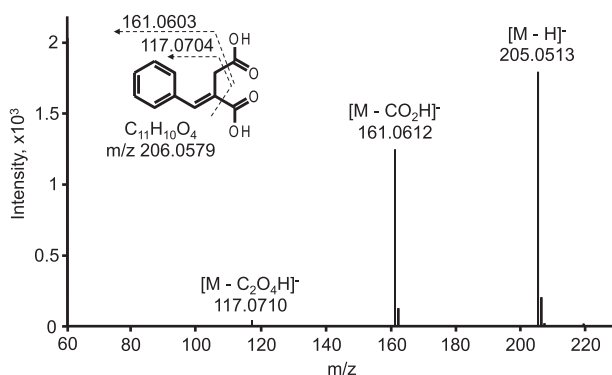


Fig. 3. The LC-MS analysis of the culture of S12-1 after the labeling reaction.

The absorbance of the developed color showed a good correlation ($R^2 = 0.9925$) with applied IA (Fig. 2D and E), indicating that the reaction transition can be quickly monitored by measurement of the absorbance after the iodine test. The results suggested that the method can be extended to high-throughput detection of IA in microbial cultures. The detection limit of the developed method was calculated as 0.13 mM. It was observed that without IA, the culture medium did not show any color change. Recently, we have reported the applicability of the coupling reaction to determine and quantify acrylic acid, a vinyl compound, in a water sample containing contaminants without any interference from the latter [16]. These findings indicated that the developed method has a high specificity against IA.

Of the 240 colonies, 37 (15.4%) showed color development after the iodine test, suggesting that these colonies produced IA-like vinyl compounds. From these, the mixture with the culture of a fungus named S12-1 was analyzed using an LC-MS system. A molecular ion at 205.0513 $[M-H]^-$ was detected, which was consistent with the calculated molecular weight of the deprotonated-labeled IA (205.0501 $[M-H]^-$) (Fig. 3). Besides, two decarboxylated anions (observed at, 161.0612 $[M-CO_2H]^-$ and 117.0710 $[M-C_2O_4H]^-$, with calculated molecular weight 161.0603 $[M-CO_2H]^-$ and 117.0704 $[M-C_2O_4H]^-$), were confirmed on the MS chromatogram. In general, decarboxylation occurs in the carboxyl groups of dicarboxylic acids having more than three carbon atoms [22]. The mass analysis showed that the S12-1 strain produced IA. The concentration of IA in the S12-1 culture in a 96-deep-well plate was found to be 0.94 mM. Sequencing of the ribosomal RNA gene revealed that S12-1 showed 99% match with *A. terreus*, thus identifying S12-1 as *A. terreus*. This finding is consistent with the fact that *A. terreus* thrives in soil [23, 24, 25].

Herein, we conclude that the findings of the present study validated through the identification of *A. terreus* could be extended to screen and identify other vital fungi producing IA by using a coupling reaction.

Declarations

Author contribution statement

Mei Sano, Yuji Aso: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Hikari Kuroda: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Hitomi Ohara, Hiroshi Ando, Keiji Matsumoto: Conceived and designed the experiments; Analyzed and interpreted the data.

Funding statement

This work was supported by JSPS KAKENHI Grant Number 19K05767 and Grant-in-Aid for JSPS Fellows Number 18J13414.

Competing interest statement

The authors declare the following conflict of interest: Hiroshi Ando and Keiji Matsumoto are employees of Kaneka Corporation.

Additional information

No additional information is available for this paper.

References

- [1] T. Cordes, A. Michelucci, K. Hiller, Itaconic acid: the surprising role of an industrial compound as a mammalian antimicrobial metabolite, *Annu. Rev. Nutr.* 35 (2015) 451–473.
- [2] K. Kato, H. Tokuda, R. Matsushima-Nishiwaki, H. Natsume, A. Kondo, Y. Ito, O. Kozawa, T. Otsuka, AMPK limits IL-1-stimulated IL-6 synthesis in osteoblasts: involvement of I κ B/NF- κ B pathway, *Cell. Signal.* 24 (2012) 1706–1712.
- [3] E.L. Mills, D.G. Ryan, H.A. Prag, D. Dikovskaya, D. Menon, Z. Zaslon, M.P. Jedrychowski, A.S.H. Costa, M. Higgins, E. Hams, J. Szpyt, M.C. Runtsch, M.S. King, J.F. McGouran, R. Fischer, B.M. Kessler, A.F. McGettrick, M.M. Hughes, R.G. Carroll, L.M. Booty, E.V. Knatko, P.J. Meakin, M.L.J. Ashford, L.K. Modis, G. Brunori, D.C. Sévin, P.G. Fallon, S.T. Caldwell, E.R.S. Kunji, E.T. Chouchani, C. Frezza, A.T. Dinkova-Kostova, R.C. Hartley, M.P. Murphy, L.A. O'Neill, Itaconate is an anti-inflammatory metabolite that activates Nrf2 via alkylation of KEAP1, *Nature* 556 (2018) 113–117.
- [4] A. Kuenz, S. Krull, Biotechnological production of itaconic acid—things you have to know, *Appl. Microbiol. Biotechnol.* 102 (2018) 3901–3914.
- [5] C.T. Calam, A.E. Oxford, H. Raistrick, Studies in the biochemistry of microorganisms: itaconic acid, a metabolic product of a strain of *Aspergillus terreus*, *Thom. Biochem. J.* 33 (1939) 1488–1495.
- [6] A. Kuenz, Y. Gallenmüller, T. Willke, K.D. Vorlop, Microbial production of itaconic acid: developing a stable platform for high product concentrations, *Appl. Microbiol. Biotechnol.* 96 (2012) 1209–1216.
- [7] K. Kinoshita, Über eine neue *Aspergillus*-Art, *Asp. itaconicus* nov. spec. *Shokubutsugaku Zasshi* 45 (1931) 45–60.
- [8] E.D. Guevarra, T. Tabuchi, Accumulation of itaconic, 2-hydroxyparaconic, itataric, and malic acids by strains of the genus *ustilago*, *Agric. Biol. Chem.* 54 (1990) 2353–2358.
- [9] T. Tabuchi, T. Sugisawa, T. Ishidori, T. Nakahara, J. Sugiyama, Itaconic acid fermentation by a yeast belonging to the genus *candida*, *Agric. Biol. Chem.* 45 (1981) 475–479.
- [10] W.F. Levinson, C.P. Kurtzman, T.M. Kuo, Production of itaconic acid by *Pseudozyma antarctica* NRRL Y-7808 under nitrogen-limited growth conditions, *Enzym. Microb. Technol.* 39 (2006) 824–827.
- [11] H. Kautola, M. Vahvaselkä, Y.-Y. Linko, P. Linko, Itaconic acid production by immobilized *Aspergillus terreus* from xylose and glucose, *Biotechnol. Lett.* 7 (1985) 167–172.
- [12] D. Kreyenschulte, E. Paciok, L. Regestein, B. Blümich, J. Büchs, Online monitoring of fermentation processes via non-invasive low-field NMR, *Biotechnol. Bioeng.* 112 (2015) 1810–1821.
- [13] E.K.R. Hanko, N.P. Minton, N. Malys, A transcription factor-based biosensor for detection of itaconic acid, *ACS Synth. Biol.* 18 (2018) 1436–1446.
- [14] M. Friedkin, Determination of itaconic acid in fermentation liquors, *Ind. Eng. Chem. Anal. Ed.* 17 (1945) 637–638.
- [15] K.F. Heck, J.P. Nolley, Palladium-catalyzed vinylic hydrogen substitution reactions with aryl, benzyl, and styryl halides, *J. Org. Chem.* 37 (1972) 2320–2322.
- [16] S. Jagtap, Heck reaction-State of the art, *Catalysts* 7 (2017) 267.
- [17] M. Sano, T. Chin, T. Takahashi, H. Ohara, Y. Aso, A simple TLC-densitometric method for the quantification of acrylic acid in aqueous solutions, *J. Planar Chromatogr. Mod. TLC* 28 (2015) 12–16.
- [18] R. Shanmugam, S. Sornambikai, N.S. Karthikeyan, K. Sathiyarayanan, A.S. Kumar, A simple colorimetric screening of nitrite using iodide in an acidic pH solution, *Austin J. Anal. Pharm. Chem.* 1 (2014) id1026.
- [19] S. Kim, E. Jung, M.J. Kim, A. Pyo, T. Palani, M.S. Eom, M.S. Han, S. Lee, A simple, fast, and easy assay for transition metal-catalyzed coupling reactions using a paper-based colorimetric iodide sensor, *Chem. Commun.* 48 (2012) 8751.
- [20] A. Hevekerl, A. Kuenz, K.-D. Vorlop, Filamentous fungi in microtiter plates—an easy way to optimize itaconic acid production with *Aspergillus terreus*, *Appl. Microbiol. Biotechnol.* 98 (2014) 6983–6989.
- [21] B.W. Jarvis, B.L. Wickes, L.M. Hoffman, Direct genomic DNA sequencing for rapid fungal identification Epic, *Biotechnol. Forum* 13 (2016) 13.
- [22] J.S. Grossert, P.D. Fancy, R.L. White, Fragmentation pathways of negative ions produced by electrospray ionization of acyclic dicarboxylic acids and derivatives, *Can. J. Chem.* 83 (2005) 1878–1890.
- [23] S.M. Lee, X.F. Li, H. Jiang, J.G. Cheng, S. Seong, H.D. Choi, B.W. Son, Terreusinone, A novel UV-A protecting dipyrroloquinone from the marine algicolic fungus *Aspergillus terreus*, *Tetrahedron Lett.* 44 (2003) 7707–7710.
- [24] J.K. Magnuson, L.L. Lasure, Organic acid production by filamentous fungi, *Adv. Fungal Biotechnol. Ind. Agric. Med.* (2004) 307–340.
- [25] B.C. Saha, Emerging biotechnologies for production of itaconic acid and its applications as a platform chemical, *J. Ind. Microbiol. Biotechnol.* 44 (2017) 303–315.