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# In Vivo Regulation of Alcohol Dehydrogenase and Lactate Dehydrogenase in *Rhizopus Oryzae* to Improve L-Lactic Acid Fermentation

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**Abstract** *Rhizopus oryzae* is becoming more important due to its ability to produce an optically pure L-lactic acid. However, fermentation by *Rhizopus* usually suffers from low yield because of production of ethanol as a byproduct. Limiting ethanol production in living immobilized *R. oryzae* by inhibition of alcohol dehydrogenase (*ADH*) was observed in shake flask fermentation. The effects of *ADH* inhibitors added into the medium on the regulation of *ADH* and lactate dehydrogenase (*LDH*) as well as the production of cell biomass, lactic acid, and ethanol were elucidated. 1,2-diazole and 2,2,2-trifluroethanol were found to be the effective inhibitors used in this study. The highest lactic acid yield of 0.47 g/g glucose was obtained when 0.01 mM 2,2,2-trifluoroethanol was present during the production phase of the pregrown *R. oryzae*. This represents about 38% increase in yield as compared with that from the simple glucose fermentation. Fungal metabolism was suppressed when iodoacetic acid, *N*-ethylmaleimide, 4,4′-dithiodipyridine, or 4-hydroxymercury benzoic acid were present. Dramatic increase in *ADH* and *LDH* activities but slight change in product yields might be explained by the inhibitors controlling enzyme activities at the pyruvate branch point. This showed that in living *R. oryzae*, the inhibitors regulated the flux through the related pathways.

**Keywords** Fermentation · Lactic acid · Ethanol · In vivo inhibition · *Rhizopus oryzae* · Alcohol dehydrogenase · Lactate dehydrogenase · Sulfhydryl reagent · Substrate/product analog

## Introduction

Lactic acid is a specialty chemical that has many applications in chemical, food, and pharmaceutical industries. Recently, there has been an expanding use of an optically pure

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lactic acid for synthesizing polylactic acid. It is expected that in the near future, the non-environmental friendly petroleum-based plastic will be substituted by polylactic acid due to its promising renewable and biodegradable properties [1–3]. Currently, lactic acid is commercially produced via fermentation because it leads to the production of the optically pure lactic acid while chemical synthesis requires drastic conditions and results in the racemates [4, 5]. To develop a more efficient lactic acid fermentation process for polylactic acid, to improve the productivity and to reduce the cost of raw materials are the major objectives [6–8]. Many microorganisms can produce lactic acid including bacteria (*Lactobacillus* and *Bacillus* species, for example) and fungi. Interestingly, the filamentous fungus *Rhizopus oryzae* can produce an optically pure L-lactic acid from simple media such as starchy materials and lignocellulosic products without prior treatment; therefore, reducing the production cost [2, 9, 10].

Unlike homofermentative lactic acid bacteria, fermentation by *R. oryzae* results in four major end products including L-lactic acid, cell biomass, fumaric acid, and ethanol. The formation of the end products depends on the cultivation conditions [11–13]. Under sufficient oxygen supply, *R. oryzae* converts glucose to pyruvate and pyruvate then enters the tricarboxylic acid (TCA) cycle where ATP and NADH are generated. The energy and cofactors produced in the TCA cycle facilitate the conversion of pyruvate to L-lactic acid by lactate dehydrogenase (*LDH*). Therefore, oxygen promotes L-lactic acid production.

Several methods have been used to prevent byproduct formation; thus, increasing L-lactic acid production. In free cell culture, high oxygen concentration is achieved by means of agitation and aeration [11, 12]. However, rigorous agitation requires high power input and leads to mycelial damage resulting in low L-lactic acid production. It was reported that improved oxygen transfer in fungal fermentation was achieved by good morphological control [14–22]. Several immobilization techniques have been used for controlling morphology including entrapment in polyurethane foam cubes or calcium alginate gels and adsorption onto the fibrous matrix or polymer supports. However, the ethanol formation as a consequence of further cell growth was still observed in long-term cultivation of those immobilized cells prepared by the techniques mentioned above. Therefore, none of them could fully limit ethanol production.

It is believed that limiting pyruvate flux toward undesirable pathways can be achieved by inhibiting the enzymes in those pathways. Previous studies revealed that ethanol was still found as the major byproduct in lactic acid fermentation by *R. oryzae* NRRL395 in the cultures with rigorous oxygen supply. Under an insufficient oxygen microenvironment, *R. oryzae* produces ethanol by the conversion of pyruvate to acetaldehyde and $CO_2$ by pyruvate decarboxylase (*PDC*) followed by the oxidation of acetaldehyde to ethanol by alcohol dehydrogenase (*ADH*). Many studies on the in vitro inhibition of yeast *ADH* (purified/partially purified) have been reported. Use of a substrate analog such as 2,2,2-trifluoroethanol or modification of *ADH* conformation by addition of sulfhydryl reagents showed significant inhibition of *ADH* activity [23–25]. Nevertheless, little is known about *R. oryzae ADH* inhibition especially in the in vivo test. In this study, suppression of *ADH* by different *ADH* inhibitors at various concentrations was elucidated in shake flask culture of living *R. oryzae*. Growth and product kinetics as well as related enzyme activities (both *ADH* and *LDH*) were investigated during fermentation. As a result of a sufficient amount of *ADH* inhibitor being present in the fermentation, it was expected that more pyruvate flux could shift toward the lactic acid production route. This would eventually lead to increasing lactic acid production.

## Materials and Methods

Lactic acid fermentation with the presence of *ADH* inhibitor was investigated in shake flask culture of immobilized *R. oryzae* on a cotton matrix (5×5 cm) in an orbital shaker operated at 30 °C, 200 rpm. The fermentation kinetics as well as the enzyme activities (both *ADH* and *LDH*) when the inhibitor was present during the fermentation were compared with those without the inhibitor (control).

### Microorganism and Inoculum Preparation

*Rhizopus oryzae* NRRL 395, a filamentous fungus producing L(+)-lactic acid obtained from the Agricultural Research Service culture collection, US Department of Agriculture, Peoria, IL, USA, was used in this study. The sporangiospores were collected from the 7-day culture on potato dextrose agar plate by shaving the surface with a sterile loop and extracting the spores with sterile water. The spore concentration was determined by spore counting using a haemacytometer. The spore suspension was diluted to $10^6$ spores/mL using sterile water.

### Growing *R. Oryzae* in the Fermentation Medium Containing Different *ADH* Inhibitors

Lactic acid fermentation by *R. oryzae* consisted of two phases, i.e. growth phase for spore germination and initial cell growth and production phase for producing lactic acid by cells obtained in the growth phase. This fermentation usually suffers from ethanol production during both growth and production phases; thus, resulting in lower lactic acid yield than the theoretical value. To inhibit ethanol production during fermentation, *ADH* inhibitors were added during the fermentation.

During the growth phase, the medium consisted of 50 g/L glucose and 5 g/L yeast extract was used for spore germination and initial cell growth where the control of pH was not necessary during this phase. Fifty milliliters of the growth medium with a preweighed cotton cloth (5×5 cm) in a 250-mL Erlenmeyer flask was inoculated with 0.5 mL *R. oryzae* spore suspension ($10^6$ spores/mL). The spores were allowed to germinate and immobilize onto the cotton matrix during the growth phase. After the growth phase (48 h), the growth medium was removed from the culture flask and 50 mL of the production medium was added for enhancing lactic acid production. The production medium contained 70 g/L glucose, 0.6 g/L $KH_2PO_4$, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.088 g/L $ZnSO_4 \cdot 7H_2O$, and 0.3 g/L urea; 5 M NaOH was added periodically for pH control during the production phase. Fermentation was carried out until glucose depletion. Samples were taken every 6 h for analyses of glucose, lactic acid, and ethanol as well as for enzyme assays. The effects of the inhibitor on fungal growth and metabolism during fermentation were investigated. Firstly, in order to compare the results from this work with the in vitro *ADH* inhibition reported in the previous works, six *ADH* inhibitors including 1,2-diazole or pyrazole (PZ), 2,2,2-trifluoroethanol (TFE), iodoacetic acid (IAA), *N*-ethylmaleimide (NEM), 4,4′-dithiodipyridine (DSDP), and 4-hydroxymercury benzoic acid or *p*-chloromercuribenzoate (PCMB) at 1 mM were initially added during the growth phase and later they were added again at the same concentration after changing the medium to the production medium [25]. The effects of those 6 inhibitors on fungal metabolism were also investigated during the production phase of the pregrown *R. oryzae* in the control growth medium (without the inhibitor). After screening those inhibitors which did not harm fungal growth and metabolism in the in vivo test, the concentration effects of such inhibitors were further studied.

## Product Yields and Volumetric Productivities

Amounts of cell growth and products formed during fermentation were analyzed. Product yield was determined from the ratio of product formed to glucose consumed during fermentation. Volumetric productivity was defined as the total amount of product formed per volume per time.

## Cell Biomass

Cell concentration in the fermentation broth was determined from the dry weight of cell biomass. Immobilized cell biomass on the cotton cloth was harvested, washed, and dried at 105 °C until reaching a constant weight. Cell concentration (g/L) was calculated by the following equation:

$$\text{Cell concentration} = \frac{\text{dry weight of immobilized cell biomasson cotton cloth (g)} - \text{dry weight of preweighed cotton cloth (g)}}{\text{volume of fermentation broth (L)}} \quad (1)$$

## Substrate and Product Analyses

High-performance liquid chromatography (HPLC) was used to analyze the organic compounds (glucose, lactic acid, fumaric acid, and ethanol) in the samples. The fermentation broth sample was centrifuged and diluted with double distilled water. The diluted particle-free sample (20 μL) was injected into an organic acid analysis column (Biorad, Aminex HPX-87 H ion exclusion organic acid column; 300×7.8 mm) maintained at 45 °C in a column oven. 0.005 M $H_2SO_4$ was used as an eluant at 0.6 mL/min flow rate. A refractive index detector was used to detect the organic compounds. A standard containing 2 g/L of each component was injected as a reference to determine the sample concentration. The peak area was used for the comparison basis. It is noted that HPLC can detect both L(+) and (D)-lactic acids.

## Cell Extraction, Partial Enzyme Purification, and Protein Determination

The sample of fresh fungal mycelia immobilized on the cotton cloth was thoroughly washed three times with sterile distilled water before eliminating the free water. The free water remaining in the sample was adsorbed onto filter papers by putting the sample in between two pieces of filter papers. Filter-dried immobilized mycelia on the cotton cloth were carefully peeled off. Two samples of 1 g filter-dried mycelia were prepared: one was for the determination of the moisture content in the filter-dried mycelia and the other was subjected to the enzyme extraction process. To determine the moisture content, 1 g sample was dried at 105 °C until reaching the constant weight. The moisture content (%) was calculated by the equation below:

$$\text{Moisture content} = \frac{\text{weight of filter} - \text{dried sample (1g)} - \text{dry weight of sample (g)}}{\text{dry weight of sample (g)}} \times 100 \quad (2)$$

Another 1 g filter-dried sample for enzyme extraction was frozen at −20 °C for 1 h before grinding in an ice-cold mortar for 5 min; 1.5 mL Tris HCl buffer solution (50 mM)

containing mercaptoethanol (10 mM) was added to the ground mycelia. The suspension of the ground mycelia with the glass beads (425–600 μm) was homogenized in an ultrasonic disruptor for 16.75 min (30-s interval with a 45-s break). Later, the glass beads were removed by filtration and the suspension was centrifuged at 12,000 rpm, 4 °C for 30 min. The supernatant, so called later the mycelial extract, was used for protein determination and enzyme assays. Protein concentration in the mycelial extract was determined by the Lowry method using bovine serum albumin as a standard [26].

### Enzyme Assays

The mycelial extract obtained from the extraction of the fresh fungal mycelia collected during fermentation with or without the inhibitors was analyzed for the activities of the enzymes of interest including *ADH* (both forward (f) and backward (b) directions) and *LDH* to investigate the consequences of the presence of the inhibitor in the living *R. oryzae* culture.

Alcohol dehydrogenase (*ADH*(b)—conversion of ethanol into acetaldehyde) was analyzed spectrophotometrically by a coupled assay system at 340 nm, depending on $NAD^+$ reduction. The reaction mixture contained 250 μmol ethanol, 4 μmol $NAD^+$, 0.2 mL mycelial extract, and 250 μmol Tris HCl buffer (pH 7.7) in a total volume of 3.15 mL [27]. The reaction was started by adding the mycelial extract into the reaction mixture. The activity was detected from the change in absorbency at 340 nm for 5 min at 30 °C (same temperature as the culture temperature). Units of *ADH*(b) activity expressed as μmol $NAD^+$ reduced per minute were determined from the calibration plot of the absorbency at 340 nm versus the concentration of $NAD^+$ in micromoles).

The conversion of acetaldehyde into ethanol was also observed by following the oxidation of NADH at 340 nm. The reaction mixture contained 250 μmol acetaldehyde, 4 μmol NADH, 0.2 mL mycelial extract, and 250 μmol Tris HCl buffer (pH 7.7) in the total volume of 3.15 mL. The reaction was started with the addition of the mycelial extract into the reaction mixture. *ADH* forward (ADH(f)) activity was detected for 5 min at 30 °C (same temperature as the culture temperature) by following the change in absorbency at 340 nm which was later converted into μmol NADH oxidized using the calibration plot (absorbency at 340 nm versus NADH concentration in μmol).

In addition, lactate dehydrogenase (*LDH*) was assayed by following the oxidation of NADH at 340 nm in the reaction mixture containing 25 μmol sodium pyruvate, 1 μmol NADH, 0.2 mL mycelial extract, and 250 μL phosphate buffer (pH 6.5) in the total volume of 3.15 mL [27, 28]. The reaction was started with the same manner as that described in the *ADH*(f) activity assay.

## Results and Discussions

### Morphology and Metabolic Responses of *R. oryzae* Grown in the Medium Containing *ADH* Inhibitors

Similar morphology was observed when culturing *R. oryzae* in the medium containing 1,2-diazole or 2,2,2-trifluoroethanol as compared with those grown in the simple glucose medium (control) (Fig. 1). Spores germinated and completely immobilized onto the cotton cloth provided. This resulted in cell-free fermentation broth. On the other hand, cell partially immobilized onto the cotton cloth when 4,4′-dithiodipyridine was present

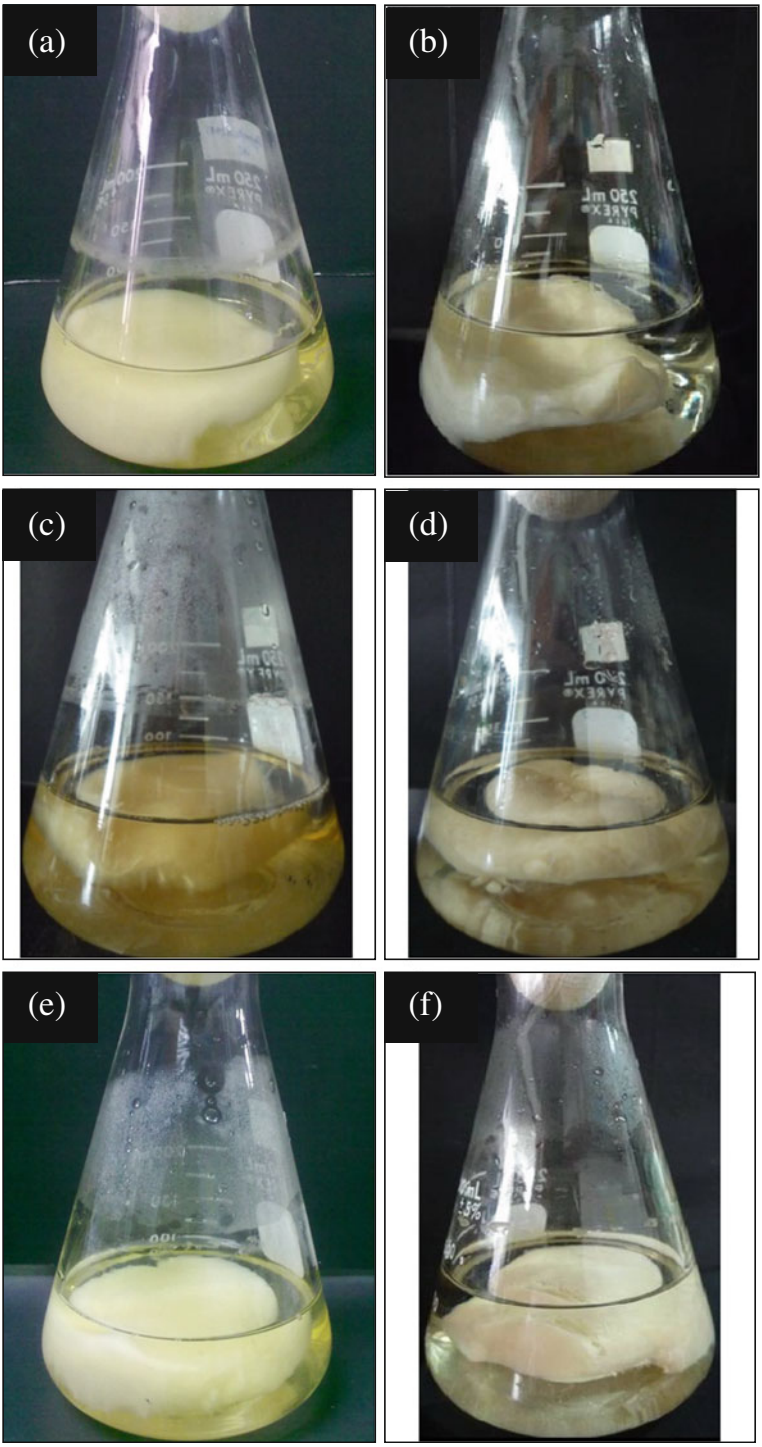
(a)
(b)
(c)
(d)
(e)
(f)

**Fig. 1** Morphology of immobilized *R. oryzae* on cotton towel grown in the medium (**a**) without the inhibitor at 24-h growth phase; **b** without the inhibitor at 24-h production phase; **c** containing 1 mM 1,2-diazole at 24-h growth phase; **d** containing 1 mM 1,2-diazole at 24-h production phase; **e** containing 1 mM 2,2,2-trifluoroethanol at 24-h growth phase; and **f** containing 1 mM 2,2,2-trifluoroethanol at 24-h production phase

during the growth phase (Fig. 2). No growth was observed in the fermentation when 4-hydroxymercury benzoic acid was added into the medium (data not shown) while prolonged growth was found in the fermentation with the presence of iodoacetic acid or *N*-ethylmaleimide in the medium during the growth phase (Fig. 2).

Table 1 shows the fermentation kinetics of *R. oryzae* cultured in the glucose medium with the presence of *ADH* inhibitor. Similar cell, lactate, and ethanol yields were obtained from fermentations with 1,2-diazole or 2,2,2-trifluoroethanol as compared with the fermentation without inhibitor during the growth phase while more cell biomass with less lactic acid and ethanol production was obtained with the presence of 4,4′-dithiodipyridine in the growth medium. Interestingly, although *ADH* inhibitor, including 1,2-diazole, 2,2,2-trifluoroethanol, or 4,4′-dithiodipyradine, was initially present since the growth phase, *R. oryzae* still produced ethanol as a major product during this phase. This was perhaps due to the affinity of these inhibitors to *ADH* at the concentration studied could not compete with the enzyme-substrate affinity under the operating conditions during the growth phase [29]. Moreover, yeast extract present in the growth medium for promoting spore germination and initial cell growth favored ethanol production simultaneously [30].

Except for the fermentations containing iodoacetic acid, *N*-ethylmaleimide, 4,4′-dithiodipyridine, or 4-hydroxymercury benzoic acid, after 48-h culture in the growth medium containing *ADH* inhibitor, the broth was discarded and replaced with fresh new production medium containing the inhibitor (1 mM). During the production phase, almost similar cell yields from the fermentations with 1,2-diazole or 2,2,2-trifluoroethanol to that from the fermentation without the inhibitor were obtained. It was evident that 1,2-diazole and 2,2,2-trifluoroethanol did not greatly affect the fermentation productivities as observed from the similar values to that of the fermentation without the inhibitor during the cultivation period. Only slightly increased lactate productivity was observed when 2,2,2-trifluoroethanol was present during the production phase. For the prolonged culture in the growth medium containing *N*-ethylmaleimide or 4,4′-dithiodipyridine, almost twice higher cell and ethanol yields as well as the productivities were observed. *R. oryzae* growth and product formation from fermentations with six *ADH* inhibitors compared with those without the inhibitor can be summarized as follows:

$$\begin{aligned}
&\text{Cell :} && \text{PCMB} < \text{IAA} < \text{NEM} < \text{CT} = \text{PZ} = \text{TFE} < \text{DSDP}\\
&\text{Lactate :} && \text{PCMB} < \text{IAA} < \text{DSDP} < \text{NEM} < \text{CT} < \text{PZ} < \text{TFE}\\
&\text{Ethanol :} && \text{PCMB} < \text{IAA} < \text{CT} = \text{PZ} = \text{TFE} < \text{NEM} < \text{DSDP}
\end{aligned}$$

Remark: the strongest inhibiting effects of the tested *ADH* inhibitor towards the strongest promoting effects on cell, lactate, and ethanol production by the inhibitor are given from the minimum to the maximum (from the left to the right). Control fermentation using the simple medium is also compared with the fermentations containing different *ADH* inhibitors in the medium.

To prevent the negative effect of *ADH* inhibitors that was found particularly in the cultures containing iodoacetic acid, *N*-ethylmaleimide, or 4-hydroxymercury benzoic acid, on initial cell growth during the growth phase, *R. oryzae* was pregrown in the simple growth medium for 48 h. During the production phase, product yields and productivities

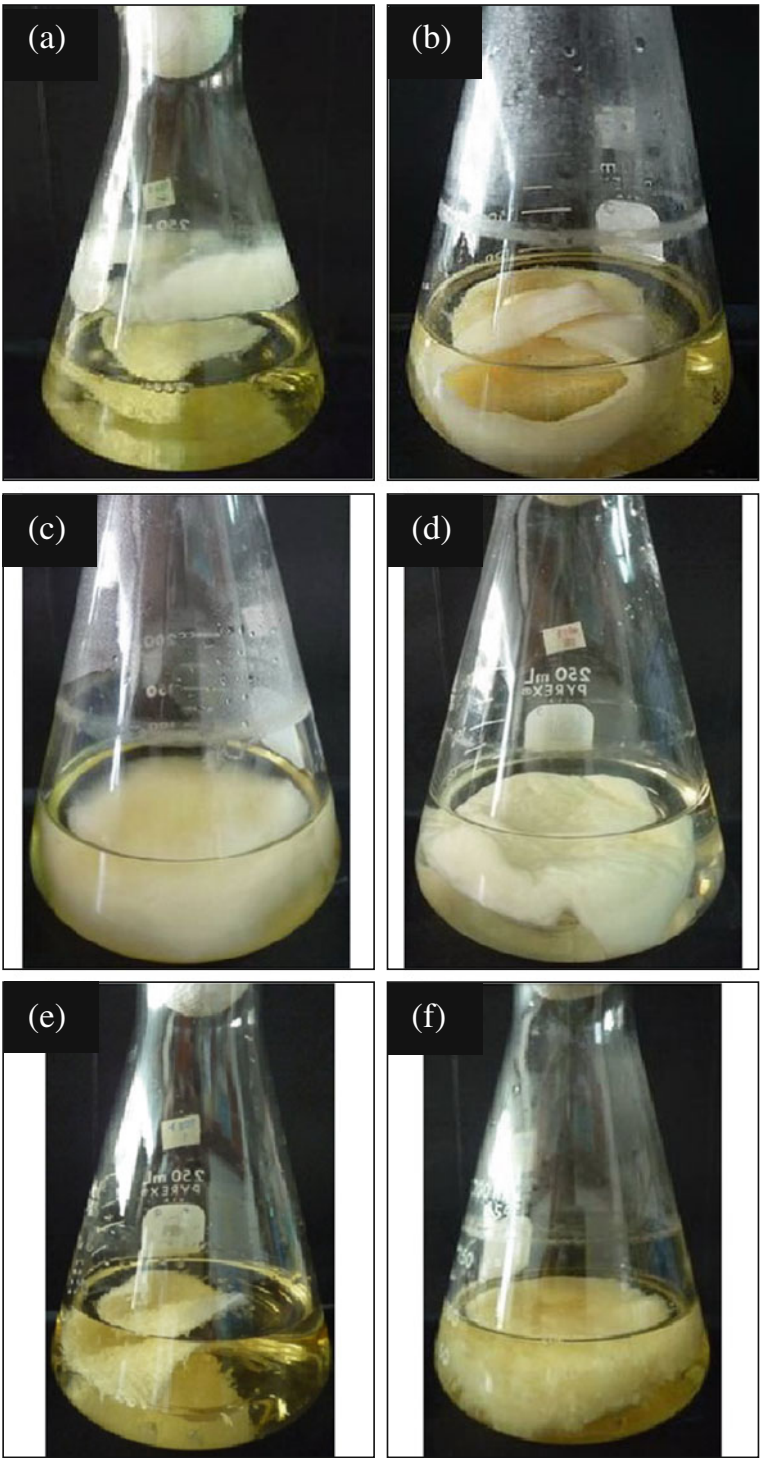
(a)
(b)
(c)
(d)
(e)
(f)

**Fig. 2** Appearance of *R. oryzae* with the presence of different *ADH* inhibitors (**a**) free cells grown in the medium containing 1 mM 4,4′-dithiodipyridine at 24-h growth phase; **b** prolonged free cell growth in the medium containing 1 mM 4,4′-dithiodipyridine at 120-h growth phase; **c** growth in the medium containing 1 M iodoacetic acid at 24-h growth phase; **d** growth in the medium containing 1 mM iodoacetic acid at 24-h production phase; **e** growth in the medium containing 1 mM *N*-ethylmaleimide at 48-h growth phase; **f** prolonged growth in the medium containing 1 mM *N*-ethylmaleimide at 120-h growth phase

correlated to the regulation of *ADH*s and *LDH* by six inhibitors were observed. Slightly increased lactate and ethanol yields compared with those of the fermentation without the inhibitor were observed in the fermentation containing 1,2-diazole or 2,2,2-trifluoroethanol while the similar cell biomass yield was obtained (Table 2). It was found that 1,2-diazole and 2,2,2-trifluoroethanol did not greatly influence the fermentation productivity during the production phase. On the other hand, iodoacetic acid, *N*-ethylmaleimide, 4,4′-dithiodipyridine, and 4-hydroxymercury benzoic acid strongly repressed *R. oryzae* metabolism as observed from low product yields and productivities. The effects of 6 *ADH* inhibitors on pregrown *R. oryzae* metabolism during the production phase can be summarized as follows:

$$\begin{aligned}
&\text{Cell}: && \text{DSDP} < \text{NEM} < \text{PCMB} < \text{CT} = \text{PZ} = \text{TFE} = \text{IAA}\\
&\text{Lactate}: && \text{PCMB} < \text{IAA} = \text{NEM} < \text{DSDP} < \text{CT} < \text{PZ} < \text{TFE}\\
&\text{Ethanol}: && \text{IAA} < \text{DSDP} < \text{NEM} < \text{PCMB} < \text{CT} < \text{TFE} < \text{PZ}
\end{aligned}$$

Remark: the strongest inhibiting effects of the tested *ADH* inhibitor towards the strongest promoting effects on cell, lactate, and ethanol production by the inhibitor are given from the minimum to the maximum (from the left to the right). Control fermentation using the simple medium is also compared along with the fermentations containing different *ADH* inhibitors in the medium.

Strong inhibitory effects of iodoacetic acid, *N*-ethylmaleimide, 4,4′-dithiodipyridine, and 4-hydroxymercury benzoic acid on ethanol production could be described from the irreversible binding of such inhibitors to the sulfhydryl group at the active site of *ADH* so that it could not serve as a ligand for the zinc atom at the catalytic site [31]. Iodoacetic acid and *N*-ethylmaleimide irreversibly alkylated the S–S bond at the active site; therefore, preventing correct conformation for substrate binding [32]. 4,4′-dithiodipyridine also irreversibly bound with the sulfhydryl group at the active site while Hg(II) complexation with the S–S bond at the active site was found with the presence of 4-hydroxymercury benzoic acid [24, 31].

In contrast to iodoacetic acid, *N*-ethylmaleimide, 4,4′-dithiodipyridine, and 4-hydroxymercury benzoic acid, 1,2-diazole (cofactor analog), and 2,2,2-trifluoroethanol (substrate/product analog) reversibly bound to the enzyme; therefore, the inhibition was competitive. Although 1,2-diazole and 2,2,2-trifluoroethanol did not exhibit significant reduction of ethanol formation they produced the little improvement in lactic acid yield when compared with the fermentation without the inhibitor. It is believed that ethanol production can be fully inhibited by the presence of the sufficient amounts of 1,2-diazole or 2,2,2-trifluoroethanol in the medium. As a result, improved lactic acid production can be accomplished.

## 1,2-Diazole and 2,2,2-Trifluoroethanol as the Competitive *ADH* Inhibitors

Competitive inhibition by 1,2-diazole or 2,2,2-trifluoroethanol was observed in the fermentations with different concentrations (0.01–10 mM) of these two inhibitors. During

**Table 1** Product yields and productivities from fermentations by *R. oryzae* in the glucose medium with the presence of *ADH* inhibitors at 1 mM during both growth and production phases

| Inhibitors | Growth phase | | | | | | Production phase | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Product yield (g/g glucose) | | | Productivity (g/L h) | | | Product yield (g/g glucose) | | | Productivity (g/L h) | | |
| | Cell | Lactate | Ethanol | Cell | Lactate | Ethanol | Cell | Lactate | Ethanol | Cell | Lactate | Ethanol |
| CT | 0.06±0.03 | 0.09±0.01 | 0.37±0.03 | 0.07±0.01 | 0.11±0.00 | 0.45±0.06 | 0.03±0.02 | 0.34±0.01 | 0.12±0.01 | 0.02±0.01 | 0.40±0.00 | 0.14±0.12 |
| PZ (1) | 0.06±0.01 | 0.10±0.01 | 0.36±0.03 | 0.07±0.01 | 0.12±0.00 | 0.43±0.07 | 0.02±0.00 | 0.37±0.01 | 0.14±0.02 | 0.02±0.00 | 0.38±0.01 | 0.15±0.02 |
| TFE (1) | 0.06±0.00 | 0.12±0.01 | 0.38±0.03 | 0.07±0.00 | 0.14±0.01 | 0.45±0.05 | 0.01±0.00 | 0.43±0.01 | 0.16±0.06 | 0.02±0.00 | 0.44±0.01 | 0.17±0.08 |
| IAA (1) | 0.01±0.00 | 0 | 0 | 0 | 0 | 0 | 0 | 0.01±0.00 | 0.06±0.00 | 0 | 0.01±0.00 | 0.04±0.00 |
| PCMB (1) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| NEM (1) | 0 | 0 | 0 | 0 | 0 | 0 | 0.05±0.00 | 0.15±0.00 | 0.27±0.00 | 0.04±0.00 | 0.16±0.00 | 0.26±0.00 |
| DSDP (1) | 0.08±0.03 | 0.06±0.00 | 0.25±0.00 | 0.03±0.00 | 0 | 0.07±0.00 | 0.04±0.00 | 0.09±0.00 | 0.29±0.00 | 0.06±0.00 | 0.10±0.00 | 0.21±0.00 |

*CT* control, *PZ* 1,2-diazole, *TFE* 2,2,2-trifluoroethanol, *PCMB* 4-hydroxymercury benzoic acid, *NEM* *N*-ethylmaleimide, *DSDP* 4,4′-dithiodipyridine, *IAA* iodoacetic acid, (1) concentration of the inhibitor at 1 mM present in the medium

**Table 2** Product yields and productivities during the production phase with the presence of *ADH* inhibitors at the concentration of 1 mM of pregrown *R. oryzae* in the simple glucose medium

| Inhibitors | Production phase | | | | | |
|---|---|---|---|---|---|---|
| | Product yield (g/g glucose) | | | Productivity (g/L h) | | |
| | Cell | Lactate | Ethanol | Cell | Lactate | Ethanol |
| CT | 0.05±0.01 | 0.34±0.01 | 0.12±0.01 | 0.02±0.01 | 0.40±0.00 | 0.14±0.01 |
| PZ (1) | 0.05±0.03 | 0.36±0.08 | 0.16±0.01 | 0.01±0.02 | 0.36±0.05 | 0.16±0.00 |
| TFE (1) | 0.05±0.02 | 0.37±0.04 | 0.15±0.05 | 0.03±0.00 | 0.40±0.03 | 0.16±0.05 |
| IAA (1) | 0.05±0.00 | 0.02±0.00 | 0.02±0.00 | 0.01±0.00 | 0 | 0 |
| PCMB (1) | 0.04±0.00 | 0 | 0.08±0.00 | 0.01±0.00 | 0 | 0.01±0.00 |
| NEM (1) | 0.01±0.00 | 0.02±0.00 | 0.06±0.00 | 0.02±0.00 | 0.003±0.00 | 0.02±0.00 |
| DSDP (1) | 0 | 0.11±0.00 | 0.04±0.00 | 0.01±0.00 | 0.12±0.00 | 0.01±0.00 |

In the growth phase, *R. oryzae* was pregrown in the simple growth medium containing 50 g/L glucose and 5 g/L yeast extract for 48 h at 30 °C and 200 rpm

*CT* control, *PZ* 1,2-diazole, *TFE* 2,2,2-trifluoroethanol, *PCMB* 4-hydroxymercury benzoic acid, NEM *N*-ethylmaleimide, *DSDP* 4,4′-dithiodipyridine, *IAA* iodoacetic acid, (1) [a] Concentration of the inhibitor at 1 mM present in the medium

the growth phase, it was found that varied concentrations of 1,2-diazole did not significantly influence the fungal metabolism as observed from the similar yields of cell biomass (~0.04–0.07 g/g glucose), lactate (~0.08–0.12 g/g glucose), and ethanol (~0.36–0.42 g/g glucose) (Table 3). But increasing lactate yield with decreasing ethanol yield was observed during the production phase when increasing 1,2-diazole concentration from 0 mM to 0.1 mM and 2,2,2-trifluoroethanol concentration from 0 to 1 mM. During the production phase, the highest lactate yield of 0.44 g/g glucose was observed from the fermentation with 0.1 mM 1,2-diazole. During the cultivation, almost similar cell biomass, lactate, and ethanol productivities were found in the fermentations with 1,2-diazole and 2,2,2-trifluoroethanol at different concentrations when compared with those in the fermentation without the inhibitor (Table 3).

The effect of 1,2-diazole and particularly 2,2,2-trifluoroethanol on lactate yield was more profound when they were added during the production phase of pregrown *R. oryzae* (Table 4). The highest lactate yield of 0.47 g/g glucose was found at the lowest 2,2,2-trifluoroethanol concentration of 0.01 mM used in this work. This was about 38% increased yield compared with that of the control fermentation (0.34 g/g glucose). Increasing 1,2-diazole concentration in the production medium slightly influenced product yields. Nevertheless, ethanol (~0.12–0.19 g/g glucose) and cell (~0.04–0.05 g/g glucose) yields from the fermentations with 1,2-diazole or 2,2,2-trifluoroethanol were not lower than those from the simple glucose fermentation as expected. It was found that productivities during the production phase were slightly affected by varying the concentration of *ADH* inhibitor (Table 4). The highest lactate productivity of 0.43 g/L h was achieved when 0.01 mM 2,2,2-trifluoroethanol was present in the production medium.

It was expected that lower ethanol yield but higher lactate yield should have been achieved from the fermentations when 1,2-diazole or 2,2,2-trifluroethanol were present; however, the kinetics data revealed that the in vivo regulation of lactic acid and ethanol production by these two inhibitors during cultivation of immobilized *R. oryzae* was not clearly seen. It could be explained that the presence of these two inhibitors in the living *R.*

**Table 3** Product yields and productivities from fermentations by *R. oryzae* in the glucose medium containing 1,2-diazole or 2,2,2-trifluoroethanol at different concentrations during both growth and production phases

| Inhibitors | Growth phase | | | | | | Production phase | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Product yield (g/g glucose) | | | Productivity (g/L h) | | | Product yield (g/g glucose) | | | Productivity (g/L-h) | | |
| | Cell | Lactate | Ethanol | Cell | Lactate | Ethanol | Cell | Lactate | Ethanol | Cell | Lactate | Ethanol |
| CT | 0.06±0.03 | 0.09±0.01 | 0.37±0.03 | 0.07±0.01 | 0.11±0.00 | 0.45±0.06 | 0.03±0.02 | 0.34±0.01 | 0.12±0.01 | 0.02±0.01 | 0.40±0.00 | 0.14±0.12 |
| PZ (0.01[a]) | 0.07±0.00 | 0.11±0.01 | 0.42±0.07 | 0.07±0.01 | 0.13±0.03 | 0.46±0.03 | 0.08±0.05 | 0.36±0.15 | 0.13±0.04 | 0.04±0.02 | 0.33±0.15 | 0.12±0.04 |
| TFE (0.01) | 0.04±0.04 | 0.09±0.03 | 0.39±0.03 | 0.07±0.01 | 0.10±0.04 | 0.41±0.00 | 0.06±0.07 | 0.32±0.14 | 0.13±0.02 | 0.01±0.00 | 0.36±0.11 | 0.15±0.00 |
| PZ (0.1) | 0.05±0.01 | 0.11±0.01 | 0.40±0.05 | 0.07±0.01 | 0.13±0.02 | 0.49±0.08 | 0.02±0.01 | 0.44±0.10 | 0.18±0.04 | 0.03±0.00 | 0.44±0.07 | 0.18±0.02 |
| TFE (0.1) | 0.06±0.00 | 0.09±0.02 | 0.40±0.03 | 0.07±0.00 | 0.12±0.02 | 0.49±0.01 | 0.04±0.03 | 0.39±0.02 | 0.17±0.01 | 0.04±0.04 | 0.43±0.09 | 0.18±0.00 |
| PZ (1) | 0.06±0.01 | 0.10±0.00 | 0.36±0.03 | 0.07±0.01 | 0.12±0.00 | 0.43±0.07 | 0.02±0.00 | 0.37±0.01 | 0.14±0.02 | 0.02±0.00 | 0.38±0.01 | 0.15±0.02 |
| TFE (1) | 0.06±0.00 | 0.12±0.01 | 0.38±0.03 | 0.07±0.00 | 0.14±0.01 | 0.45±0.05 | 0.01±0.00 | 0.43±0.01 | 0.16±0.06 | 0.02±0.00 | 0.44±0.01 | 0.17±0.08 |
| PZ (10) | 0.05±0.00 | 0.11±0.02 | 0.41±0.02 | 0.06±0.01 | 0.12±0.02 | 0.49±0.06 | 0.01±0.01 | 0.32±0.07 | 0.19±0.01 | 0.01±0.01 | 0.33±0.05 | 0.19±0.00 |
| TFE (10) | 0.06±0.01 | 0.08±0.01 | 0.39±0.10 | 0.06±0.01 | 0.09±0.03 | 0.42±0.02 | 0.02±0.02 | 0.33±0.03 | 0.16±0.03 | 0.02±0.02 | 0.29±0.02 | 0.14±0.05 |

*CT* control, *PZ* 1,2-diazole, *TFE* 2,2,2-trifluoroethanol

[a] Concentration of the inhibitor in millimolars present in the medium

**Table 4** Product yields and productivities during the production phase with the presence of various concentrations of 1,2-diazole or 2,2,2-trifluoroethanol of pregrown *R. oryzae* in the simple glucose medium

| Inhibitors | Production phase | | | | | |
|---|---|---|---|---|---|---|
| | Product yield (g/g glucose) | | | Productivity (g/L-h) | | |
| | Cell | Lactate | Ethanol | Cell | Lactate | Ethanol |
| CT | 0.05±0.01 | 0.34±0.01 | 0.12±0.01 | 0.02±0.01 | 0.40±0.00 | 0.14±0.01 |
| PZ (0.01[a]) | 0.04±0.02 | 0.37±0.08 | 0.14±0.07 | 0.01±0.01 | 0.37±0.06 | 0.15±0.08 |
| TFE (0.01) | 0.04±0.02 | 0.47±0.03 | 0.15±0.03 | 0.02±0.01 | 0.43±0.00 | 0.14±0.02 |
| PZ (0.1) | 0.04±0.03 | 0.38±0.04 | 0.16±0.01 | 0.01±0.01 | 0.37±0.06 | 0.16±0.01 |
| TFE (0.1) | 0.04±0.03 | 0.42±0.01 | 0.19±0.03 | 0.04±0.03 | 0.42±0.04 | 0.19±0.02 |
| PZ (1) | 0.05±0.03 | 0.36±0.08 | 0.16±0.01 | 0.01±0.02 | 0.36±0.05 | 0.16±0.00 |
| TFE (1) | 0.05±0.02 | 0.37±0.04 | 0.15±0.05 | 0.03±0.00 | 0.40±0.03 | 0.16±0.05 |
| PZ (10) | 0.04±0.04 | 0.34±0.01 | 0.19±0.06 | 0.07±0.06 | 0.35±0.00 | 0.19±0.05 |
| TFE (10) | 0.04±0.04 | 0.32±0.00 | 0.13±0.01 | 0.02±0.00 | 0.34±0.02 | 0.13±0.01 |

In the growth phase, *R. oryzae* was pregrown in the simple growth medium containing 50 g/L glucose and 5 g/L yeast extract for 48 h at 30 °C and 200 rpm

*CT* control, *PZ* 1,2-diazole, *TFE* 2,2,2-trifluoroethanol

[a] Concentration of the inhibitor in millimolars present in the medium

*oryzae* culture with complex enzyme system caused metabolic shift not only at the targeted alcohol fermentation pathway as previously expected. Better understanding in metabolic flux balance when these two inhibitors are present in the living *R. oryzae* culture is required in order to achieve limited ethanol production and enhanced lactic acid production.

## In Vivo Effects of *ADH* Inhibitors on Enzyme Activities during Lactic Acid Fermentation

Figure 3 represents changes in *LDH* and *ADH* activities throughout the cultivation period when 1,2-diazole or 2,2,2-trifluoroethanol were present in both growth and production phases. During the growth phase (0–48 h), enzyme activities increased with the increasing time. The enzyme activities continuously dropped during the production phase (48–120 h). The highest activities were found at 48-h cultivation. Similar results were investigated by Chotisubha-anandha and coworkers [33]. They observed the increase in the activity of *LDH* with the increasing fermentation time (from 0 to 48 h). Later, the activity dropped due to glucose depletion. When 2,2,2-trifluoroethanol was present, the activities of *LDH, ADH(f), and ADH(b)* were dramatically increased while similar activities were found in the fermentation with 1,2-diazole when compared with those in the fermentation in the simple glucose medium. The increasing enzyme activities when 2,2,2-trifluoroethanol was added into the culture is presumably explained by the analogous chemical structures of 2,2,2-trifluoroethanol and ethanol. This might lead to the increasing enzyme expression level due to cellular response to the substrate/product analog. In case of 1,2-diazole, it competes with the cofactors $NAD^+$/NADH; thus, it does not directly influence the enzyme expression in the living *R. oryzae* culture. Low enzyme activities were found in the fermentations containing iodoacetic acid, *N*-ethylmaleimide, 4,4′-dithiodipyridine, and 4-hydroxymercury benzoic acid along the cultivation period (data not shown).

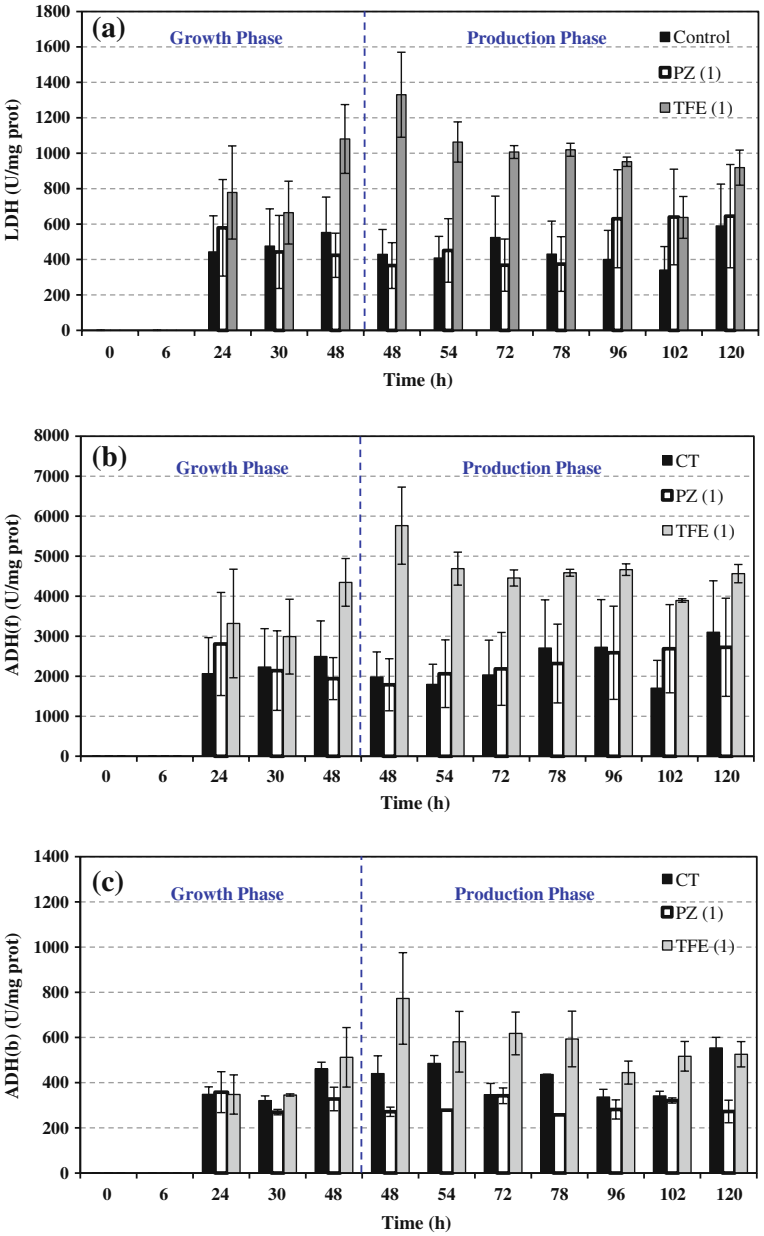


**Fig. 3** Enzyme activities during the fermentation by *R. oryzae* in the glucose medium containing 1,2-diazole (*PZ*) or 2,2,2-trifluoroethanol (*TFE*) at 1 mM; **a** lactate dehydrogenase (*LDH*); **b** alcohol dehydrogenase (*ADH(f)*)—acetaldehyde to ethanol; and **c** alcohol dehydrogenase (*ADH(b)*)—ethanol to acetaldehyde

Similar trends of changes in enzyme activities were found in the fermentations of pregrown *R. oryzae* compared with those fermentations when the inhibitors were added during the growth phase and that without the inhibitor. Firstly, *R. oryzae* was pregrown in

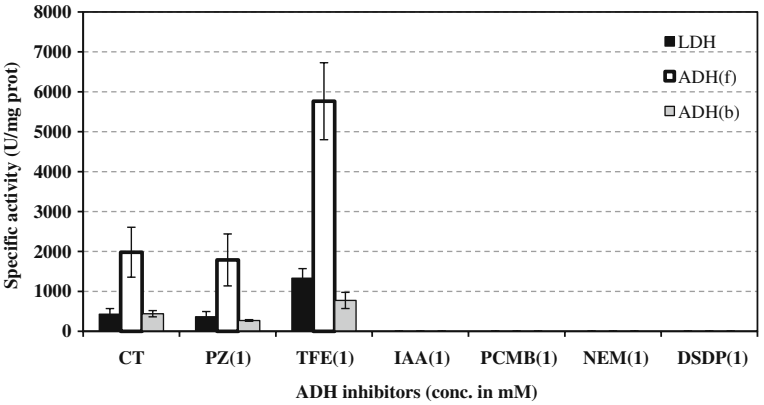


**Fig. 4** Activities of lactate dehydrogenase (*LDH*) and alcohol dehydrogenase (both *ADH(f)* and *ADH(b)*) at 48 h. During growth and production phases, *R. oryzae* was cultivated in the medium containing various *ADH* inhibitors at 1 mM

the simple glucose medium during the growth phase. Later in the production phase the inhibitor was added into the medium. Figure 4 shows the enzyme activities at 48-h cultivation. No enzyme activity was detected when iodoacetic acid, *N*-ethylmaleimide, 4,4′-dithiodipyridine, or 4-hydroxymercury benzoic acid were added into the production medium. While the fermentation with 2,2,2-trifluoroethanol led to increasing *LDH* and *ADH*s activities. The presence of 1,2-diazole in the medium did not seem to affect the enzyme expression (Fig. 4). As stated in the previous studies, the binding of iodoacetic acid, *N*-ethylmaleimide, 4,4′-dithiodipyridine, or 4-hydroxymercury benzoic acid to the enzyme active site are irreversible [23–25]. Therefore, the presence of these four inhibitors during cultivation of living *R. oryzae* prevented further enzyme-substrate binding. This eventually led to no enzyme activity found when performing the enzyme assays of the mycelial extract later.

It is obvious that the addition of the inhibitor at an early stage of the fermentation caused metabolic repression (Figs. 4 and 5). When the inhibitor was added only during the production phase, higher enzyme expression levels were found compared with those with the inhibitors since the growth phase (Figs. 4 and 5). Almost 50 times increased *LDH* activity was observed during the production phase with the presence of 2,2,2-trifluoroethanol of *R. oryzae* culture that was pregrown in the simple glucose medium. Although *ADH(f)* activity was lower than *LDH* activity in the fermentations containing the inhibitors, it was still higher than that found in the control fermentation. The increasing enzyme activity when the inhibitor was present only in the production phase suggested that the addition of *ADH* inhibitor during the production phase is sufficient for improving lactic acid production. Also, the chance of cell damage by adding the inhibitor only in the production phase can be prevented.

In this study, the inhibition of *ADH* by the inhibitors associated with the lower *ADH* activity and eventually led to less ethanol produced during the cultivation of *R. oryzae* was expected. But in the living cells, the addition of the potential *ADH* inhibitors led to highly increasing specific enzyme activities of *LDH* and *ADH*s compared with the specific activities observed in the glucose fermentation without the inhibitor (control). These results are presumably explained by the influence of the inhibitor added as well as

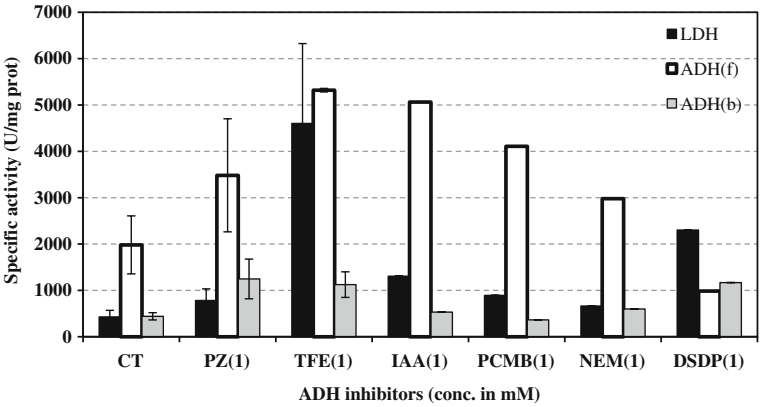


**Fig. 5** Activities of lactate dehydrogenase (*LDH*) and alcohol dehydrogenase (both *ADH(f)* and *ADH(b)*) at 48 h with various *ADH* inhibitors at 1 mM. During the growth phase, *R. oryzae* was pregrown at the simple glucose medium at 30 °C, 200 rpm

the effects of other environmental factors such as local nutrient/oxygen transport. For example, for all cases (with or without the inhibitors) at some local area likely at the central core of the immobilized cell matrix, oxygen might be limited; thus, *R. oryzae* cells in such an area readily entered alcohol fermentative pathway. With the presence of the inhibitor, some *ADH*s produced were bound with the inhibitor reversibly or irreversibly depending on the type of inhibition. Presumably, cells responded to this phenomenon by increasing *ADH* expression. As a consequence, largely increased *ADH* activities were observed as compared with those in the control cultivation. Increasing *LDH* specific activity was also observed. This might be explained by the metabolic shift at pyruvate branch point when the *ADH* inhibitor was present because *ADH* inhibitors controlled flows through all the metabolic routes. The concentrations of these inhibitors were the regulating signals which governed the activities of the controlling enzymes at the pyruvate branch point.

## Conclusions

This study reveals that ethanol production dominated during the growth phase while lactic acid production took over during the production phase. The presence of *ADH* inhibitors earlier during the growth phase gave strong inhibition of cell growth and metabolism of *R. oryzae*. Slightly increasing lactic acid production but significant increasing *LDH* activity was observed in the fermentation containing 2,2,2-trifluoroethanol during the production phase. In addition, *ADH* activities in the fermentation with 2,2,2-trifluoroethanol were dramatically increased but the similar ethanol production in the fermentation without the inhibitor was obtained. It is suggested that with the presence of *ADH* inhibitor, less pyruvate shifts toward ethanol production; thus, an increase in lactic acid or cell biomass production should have been observed. However, the results shown in this study implied that in vivo inhibition of *ADH* by adding the inhibitors during fermentation did not only alter the conversion of acetaldehyde to ethanol but it also shifted the pyruvate flux through other related pathway.

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