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Effect of redox potential on the growth of *Yarrowia lipolytica* and the biosynthesis and activity of heterologous hydroperoxide lyase

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Abstract

The aim of this study was to investigate the effect of redox potential (Eh) on the growth of the yeast *Yarrowia lipolytica* in both oxidizing (Eh = +350 mV) and reducing (Eh = −150 mV) media and its effect on the expression and activity of hydroperoxide lyase (HPL). HPL activity was assayed in media with Eh values ranging from −250 to +720 mV. In order to change the Eh value of the media, reducing agents including dithiotreitol (1 g/L) and hydrogen (4%) as well as oxidants such as potassium ferricyanide (1 g/L) and oxygen (100%), were used. The experimental findings showed that oxidizing conditions, with Eh of +350 mV, were favorable for the growth of the yeast, whereas reducing conditions, with Eh values of −150 mV, resulted in a higher expression of HPL. In addition, the results showed that the enzymatic activity of the purified HPL was enhanced in the presence of 0.5 mM dithiotreitol but decreased with 1 mM potassium ferricyanide and bubbling O_2. However, HPL activity increased 1.5 times in the presence of 4% hydrogen with an Eh value of −170 mV.

Keywords: *Yarrowia lipolytica*; Culture medium; Oxido-reduction potential (ORP); Hydroperoxide lyase activity

1. Introduction

Hydroperoxide lyase (HPL) is an enzyme widely distributed in plants and involved in the biosynthesis of volatile aldehydes and ω-oxo-acids, obtained by cleavage of the C–C bond between the hydroperoxide (HP) group and neighboring double bond of HPs of polyunsaturated fatty acids (PUFAs), such as linoleic acid [1]. Aldehydes (C6 and C9) as well as their corresponding alcohols, obtained from the HPL bioconversion of 13- and 9-HPs of linoleic and linolenic acids, respectively, are important constituents of the characteristic flavors of fruits, vegetables and green leaves [2]. These flavoring compounds are molecules of higher added-value, widely used in the aroma industry.

Previous work by our group [3] showed that the chloroplast fraction and purified HPL from green bell pepper were of particular interest for the production of green note molecules, such as hexanal and *trans*-2-hexenal. However, the amount of enzyme recovered was limited, making the cost of biocatalyst production too high to be considered for an industrial application.

Subsequent research work carried out by our group [4] was aimed at the genetic expression of HPL from green bell pepper in the yeast *Yarrowia lipolytica*. The HPL activity from the yeast extract as well as the whole yeast cells was measured to be 1200 enzyme U/L culture medium. In addition, the amount of C6-aldehydes produced directly in the culture medium, after addition of the HP substrates, was relatively high (350 mg/L reaction medium). A process using whole *S. cerevisiae* cells transformed with banana HPL was previously developed to produce *cis*-3-hexenal and *cis*-3-hexenol [5].

The literature reported that micro-organisms were sensitive to the oxido-reduction potential (ORP) of the culture medium [6]. The ORP has also been called the redox potential (Eh) and is a physicochemical parameter that determines the oxidizing or reducing properties of the medium. Eh depends essentially upon the composition of the medium in terms of the properties

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of compounds such as proteins, peptides, thiol-containing amino acids and reducing sugar as well as medium pH, temperature and, for a large part, the concentration of soluble oxygen. Eh also plays an important role in the cellular physiology of micro-organisms such as growth capacity [7,8], enzyme expression [9] and thermal resistance [10–12].

The literature indicated that control of ORP enhanced the production of amino acids by *Corynebacterium glutamicum* [13] and xylitol by the yeast *Candida parapsilosis* [14]. Wang et al. [15] reported a beneficial effect in the use of reduced media for the production of γ-decalactone by the yeast *Sporidiobolus ruinenii*. Hence, ORP could be an appropriate parameter, controlled by adjusting the Eh, for the production of aroma compounds by micro-organisms.

The present research is part of on-going work by our group [16–18] aimed at the development of an approach for the biotechnological production of aroma compounds by *Y. lipolytica*. The specific objective of the present work was to investigate the effect of a wide range of Ehs on the growth of *Y. lipolytica* as well as on the expression and activity of HPL.

2. Experimental

2.1. Yeast culture media

The transformed *Y. lipolytica* strain JMY 861, possessing a 6-His-tagged green bell pepper HPL, was grown at 27 °C for 48 h on YTGA medium containing 5 g/L yeast extract, 10 g/L tryptone, 10 g/L glucose and 15 g/L agar. The biomass was harvested and re-suspended in physiologic water (9% NaCl) which was in turn incubated at 27 °C for 24 h in 300 mL YTG medium containing 5 g/L yeast extract, 5 g/L tryptone and 10 g/L glucose at an initial optical density (OD) of 0.2 at λ_{600}. The biomass was then incubated at an initial OD_{600} of 4.0 (10^7 cells/mL) in YTO medium containing 5 g/L yeast extract, 10 g/L tryptone, 10 g/L olive oil, 5.3 g/L NH_4Cl and either 1 g/L potassium ferricyanide ($K_3Fe(CN)_6$) as the oxidant molecule, 1 g/L dithiotreitol (DTT) as the reducing molecule or under a flux of 4% H_2 (0.1 vvm). The biomass was cultivated at an agitation of 140 rpm and at 27 °C, in a Biostat Q multifermentor (B-Braun-Biotec International, Melsungen, Germany), equipped with pH, Eh, pO_2, temperature and agitation measurement units. Data were obtained with a DCU Interface, equipped with MFCS Win 2.0 software (B-Braun-Biotech International). The biomasses, harvested at different time periods of fermentation, ranging from 0 to 96 h, were used throughout this study.

2.2. Enzyme extraction

The harvested biomass was washed three times with Tris–HCl buffer solution (0.1 M, pH 8.0) and the washed cells were suspended (1/6, w/v) in Tris–HCl buffer solution containing 2% Triton X-100R (Sigma Chemical Co., St-Louis, MO). The suspended cells were homogenized, with a one-shot cell disrupter (Z plus series "One shot") at 1.6 kbar, and centrifuged three times (10,000 × *g*, 10 min). The resulted supernatant was considered to be the enzymatic crude extract.

2.3. Protein determination

The protein content of the enzymatic fractions was determined according to a modification of the Lowry method [19], using bovine serum albumin (Sigma Chemical Co.) as a standard for calibration.

2.4. Substrate preparation

Hydroperoxides were prepared according to the procedures described by Gargouri et al. [20] by oxidation of 500 mg of linoleic acid (Sigma Chemical Co.), using 15 mg soybean lipoxygenase (Sigma Chemical Co.; 45,000 U/mg solid) in borate buffer solution (0.1 M, pH 9.6). After 1 h of incubation (25 °C, 300 rpm, 30 mL O_2/min), the enzyme was inactivated by adjusting the pH to 4.0 with 96% H_2SO_4. The linoleic acid hydroperoxides (HPODs) were extracted three times with diethyl ether (1/1, v/v) and any traces of water were eliminated by the addition of dry $MgSO_4$ (Sigma Chemical Co.). The organic solvent was removed by evaporation under vacuum and the dried HPs were solubilized in absolute ethanol. The substrate stock was then stored at −20 °C. The total bioconversion of fatty acids into HPs was monitored by measuring the absorbance at 234 nm.

2.5. Protein purification

For HPL purification of the crude enzymatic extract, yeast cells were suspended in Tris–HCl buffer (0.1 M, pH 8.0) containing 2% Triton X-100R, 0.3 M NaCl, 0.01 M imidazole and a protease inhibitor EDTA-free cocktail (one cocktail tablet for 50 mL of Tris–HCl buffer) (Roche Diagnostics, Penzberg, Germany) consisting of 0.02 mg/mL pancreas-extract, 0.002% mg/mL chymotrypsin, 0.005 mg/mL thermolysin, 0.02 mg/mL trypsin and 0.33 mg/mL papain. A Ni-NTA Agarose resin column (1 cm × 1 cm) (Quiagen, Courtaboeuf, France) was equilibrated, at a flow rate of 0.5 mL/min, with 20 mL of buffer A (Tris–HCl, 0.1 M, pH 8.0) containing 0.2% Triton X-100R, 0.3 M NaCl and 0.01 M imidazole. The crude enzymatic extract (25 mL) was loaded onto the column at a flow rate of 0.3 mL/min and the resin was subsequently washed with 16 mL of buffer B (sodium phosphate, 0.05 M, pH 6) containing 0.2% Triton X-100R, 0.3 M NaCl and 0.015 M imidazole at a flow rate of 0.5 mL/min. The His-tagged protein was eluted with 50 mL buffer B containing 0.25 M imidazole. All steps were carried out at 4 °C.

2.6. Enzymatic assay

The HPL activity of the crude enzymatic extract, obtained from biomasses grown on the selected media, was determined in MES–KOH buffer (0.2 M, pH 5.5) containing 0.025 M of the 13-HP stock solution. The enzymatic reaction was initiated by the addition of 4 μL of the enzymatic extract. The decrease in absorbance at λ_{234}, due to the bioconversion of fatty acid HPs into corresponding aldehydes, was followed spectrophotometrically during a 1 min period and activity was calculated from the initial slope of the resulting absorbance plot. For the purified HPL, the enzymatic assay was performed in the presence of

different concentrations of $K_3Fe(CN)_6$ or DTT. In order to have different Eh values in the reaction media, H_2 or O_2 fluxes were used for a wide range of time periods. One unit of HPL activity was defined as the amount of enzyme that converted 1 μmol substrate into its respective end product in 1 min of time.

3. Results and discussion

3.1. Effect of Eh on the growth of Y. lipolytica

The dried biomass of *Y. lipolytica* was measured at 24 h intervals during the growth phase in the selected media, including a control medium, a medium containing 1 g/L DTT, a medium containing 1 g/L $K_3Fe(CN)_6$ and a medium with 4% H_2.

Fig. 1A shows that the highest biomass yield (2.3 g/L) was obtained after 24 h in the control medium (Eh = +300 mV, Fig. 2). Media containing either $K_3Fe(CN)_6$ with oxidizing conditions of +420 mV or H_2 with reducing conditions of −100 mV showed the same profiles with maximum biomass yields of 1.8 g/L, after a 72 h culture period. The lowest biomass yield (1 g/L) was obtained with the medium containing DTT (−200 mV).

Figs. 2 and 3 show the effect of different Eh and pO_2 values, respectively, of the selected media on *Y. lipolytica* growth. The experimental findings indicate that the lower the Eh of the medium, the slower the growth of *Y. lipolytica*, so that maximum biomass yield was found to be in the control medium. Media containing either $K_3Fe(CN)_6$ or H_2 exhibited Eh value ranging from 0 to −100 mV and were found to have no influence on the growth of the yeast. However, in the two media containing either $K_3Fe(CN)_6$ or H_2, yeast growth was less important and pO_2 decreased less rapidly than that observed in the control medium. The decrease in Eh values during the first 12 h of microbial growth period could have been due to the uptake of oxidizing compounds such as soluble O_2 as well as the release of intermediate metabolites, possessing a reducing capacity, into the culture medium.

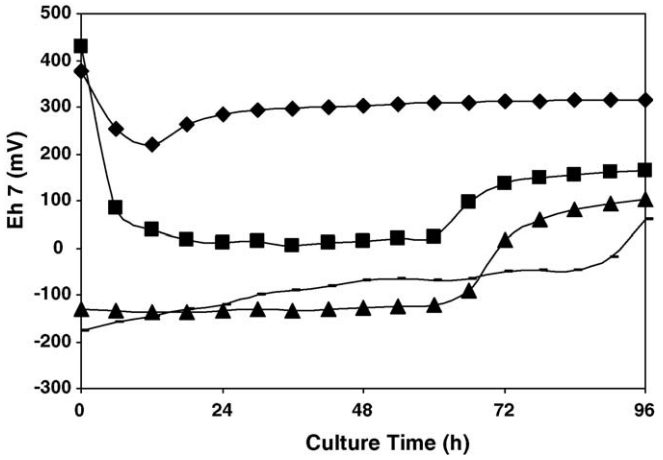

Fig. 2. Eh evolution during the growth of *Yarrowia lipolytica* in the Biostat Q fermentor in the four media: (♦) control, (■) $K_3Fe(CN)_6$, (▬) DTT and (▲) H_2.

The medium containing DTT showed (Fig. 2) the same Eh as that obtained with 4% H_2 (−100 mV) during the whole period of culture growth. However, biomass yield was two times lower in the medium containing DTT compared to the medium containing H_2. The overall results suggest that the reducing molecule DTT played an active role in trapping soluble O_2 as confirmed by the stable pO_2 values (50% of saturation) during the entire culture period. Although the results (Figs. 1A and 2) show that the Eh of the culture medium had a great influence on the growth of the yeast, the molecular effect of DTT could not be discarded.

These experimental findings (Fig. 1A) are in agreement with those reported in literature. Georges et al. [11] indicated that *Escherichia coli* was able to grow better in oxidizing conditions

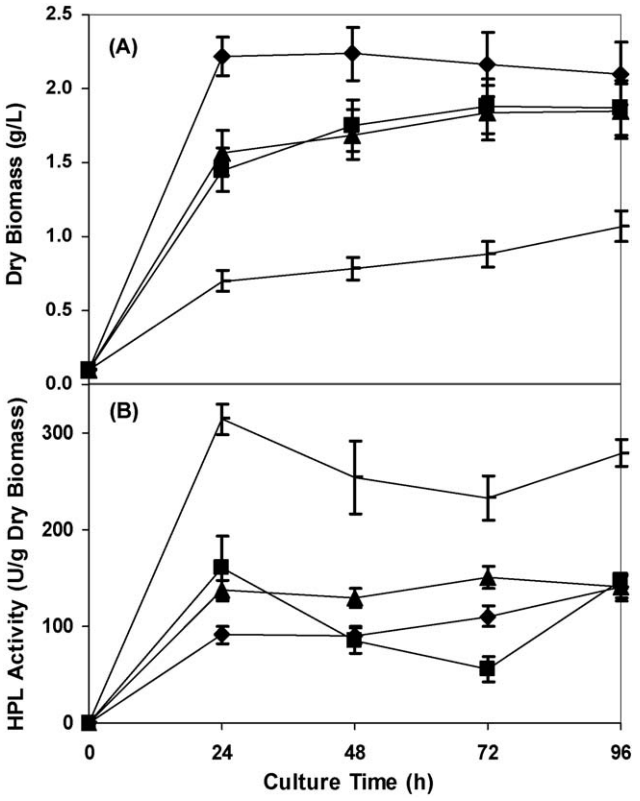

Fig. 1. (A) Biomass evolution and (B) HPL activity during culture incubation of *Yarrowia lipolytica* in the Biostat Q fermentor for the four different media: (♦) control, (■) $K_3Fe(CN)_6$, (▬) DTT and (▲) H_2.

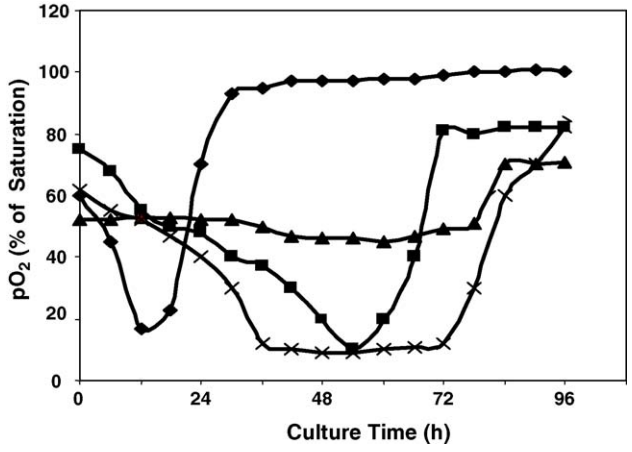

Fig. 3. pO_2 evolution during the growth of *Yarrowia lipolytica* in the Biostat Q fermentor in the four media: (♦) control, (■) $K_3Fe(CN)_6$, (▲) DTT and (×) H_2.

compared to its growth in reducing ones. Riondet et al. [21] demonstrated that the reduced ORP of the culture medium of *E. coli* induced a decrease in the proton motive force of the bacteria which, in turn, induced an increase in their permeability to protons resulting in a decrease in the intracellular pH.

3.2. Effect of Eh on the biosynthesis of HPL

The biosynthesis of HPL was maximal at 24 h of culture incubation with biomass grown on the reduced medium containing DTT (Fig. 1B). The results showed the presence of more than 300 U/g biomass, which was three times more than that obtained with biomass grown in the control medium (100 U/g biomass) and two times greater than that with biomass grown on oxidizing and reducing media (150 U/g biomass).

Experimental findings in our laboratory (unpublished data) showed a beneficial effect in the use of the reduced medium on the production of γ-decalactone by the yeast *Sporidiobolus*. Riondet et al. [12] reported that the comparison of *E. coli* fermentations at −100 and −320 mV showed that metabolic fluxes were diverted with different proportions of end products being obtained; these authors assumed that this metabolic deviation was due to the variation of the specific activity of the enzymes implicated in fermentation metabolism.

In addition, the extracellar ORP could have modified a number of molecules in the cell at their active sites, such as the thiol, amino acyl, flavin and quinone groups [22]. Suh et al. [23] showed that the flavine mono-oxygenase (FMO) activity was ORP dependant since FMO was active in reduced conditions and inhibited in the presence of oxidant ORP. These authors [22] suggested that cystein 353 and 339 of FMO were responsible for the regulation of the intracellular ORP; these two amino-acyl residues could have formed an intramolecular disulfide bridge thereby blocking access of the substrate to the active site, hence decreasing the activity of the enzyme.

On the other hand, the results (Fig. 2) show that the presence of 1 g/L DDT in the culture medium induced a decrease of the Eh value to −100 mV without any concomitant decrease in cell viability (100% survival). DTT is a reducing and permeabilizing agent known to provide a reduced intracellular environment favorable for the formation of disulfide bonds [24]; the conformation of proteins and enzyme activity could therefore have been affected by the local Eh. These experimental findings (Fig. 1B) suggest that HPL was activated by a direct effect of DTT through its role as antioxidant where it protected the thiol function at the active site of the enzyme.

Moreover, the experimental results (data not shown) demonstrated that the specific activity of HPL (1.6 U/mg protein) was higher for the yeast grown on DTT medium (Eh −100 mV) than that reported in literature for green bell pepper HPL (0.15–0.35 U/mg protein) [25] and tomato HPL (0.169 U/mg protein) [26].

3.3. Effect of Eh on the specific activity of purified HPL

The effect of Eh on the specific activity of purified HPL was investigated in the presence of reducing DDT or oxidizing $K_3Fe(CN)_6$ molecules as well as gaz fluxes of 4% H_2 and pure O_2.

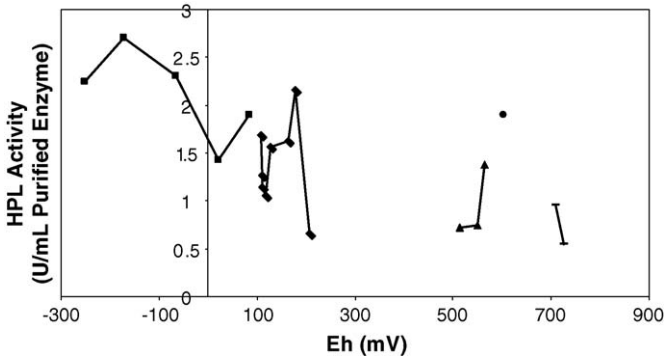

Fig. 4. Effect of Eh on the activity of the purified HPL in four assay media: (●) control, (▲) $K_3Fe(CN)_6$, (♦) DTT, (■) H_2, and (▬) O_2. Experiments were performed in triplicate but only one trial is represented.

The concentrations of DTT and $K_3Fe(CN)_6$ that were investigated varied from 0.1 to 25 mM and from 0.1 to 1 mM, respectively. Fig. 4 shows the effect of the Eh of the media on HPL activity. The HPL activity for the control trial was 1.9 U/mL at an Eh of +600 mV. The presence of 0.1–1.0 mM $K_3Fe(CN)_6$ induced a decrease in the Eh value to +510 mV and a decrease in HPL activity ranging from 0.7 to 1.4 U/mL. Using bubbling O_2 in the assay medium, a higher Eh of +709 to +727 mV and lower HPL activity of 1–0.6 U/mL were obtained compared to those obtained in the control trial. These results suggest that there were some interactions between the oxidant molecules and the fatty acid HP substrate; however, the mechanism of these interactions was still not elucidated and, in a more general term, the oxidant conditions (+500 to +720 mV) were not optimal for HPL activity.

In more reduced conditions (−250 to +200 mV), two phenomena could be cited. The first one was related to the reducing effect of DDT, which decreased the Eh (+107 to +207 mV), while the second one was the interference of DDT with the substrate and enzyme. The use of 0.5 mM DTT produced an optimum Eh of +178 mV which slightly enhanced the HPL activity (2.16 U/mL) compared to that of the control. However, higher concentrations of DTT (1–25 mM) resulted in a decrease in the Eh values, up to +107 mV, as well as HPL activity which could be attributed to the molecular effect of DTT on HPL. These experimental findings (Fig. 4) are in agreement with those reported in literature. Matsui et al. [27] and Shibata et al. [2] studied the effect of antioxidants and reported that these molecules, at concentrations up to 20 mM, showed an inhibitory effect on HPL activity by capturing intermediate radicals [28], present at high concentrations, responsible for the cleavage of HPs thereby resulting in a decrease of HPL activity.

The highest HPL activity (2.7 U/mL) was obtained in much more reduced conditions (−170 mV) during the bubbling of H_2 into the reaction medium. These results suggest the absence of any molecular effect of H_2; however, Eh values may have increased the HPL activity by 1.5-fold compared to that obtained in the control trial. Laurinavichene et al. [29] also demonstrated an increase in hydrogenase-2 biosynthesis in *E. coli* in the presence of H_2.

4. Conclusion

The results gathered in this study show that oxiding conditions were favorable for the growth of *Y. lipolytica*, whereas reducing conditions were more appropriate for the biosynthesis of HPL. In addition, the activity of purified HPL was enhanced in an assay medium that was reduced with bubbling H_2. HPL activity was also affected not only by the Eh value but by the molecules of the reaction medium. It was therefore important to avoid the molecular effect of these reagents by using gazes when the ORP was investigated.

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