Fungal and Bacterial Physiology

Application of *n*-dodecane as an oxygen vector to enhance the activity of fumarase in recombinant *Escherichia coli*: role of intracellular microenvironment

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ABSTRACT

The effect of the intracellular microenvironment in the presence of an oxygen vector during expression of a fusion protein in *Escherichia coli* was studied. Three organic solutions at different concentration were chosen as oxygen vectors for fumarase expression. The addition of *n*-dodecane did not induce a significant change in the expression of fumarase, while the activity of fumarase increased significantly to 124% at 2.5% *n*-dodecane added after 9 h induction. The concentration of ATP increased sharply during the first 6 h of induction, to a value 7600% higher than that in the absence of an oxygen-vector. NAD/NADH and NADP/NADPH ratios were positively correlated with fumarase activity. *n*-Dodecane can be used to increase the concentration of ATP and change the energy metabolic pathway, providing sufficient energy for fumarase folding.

Introduction

Molecular oxygen is an environmental and developmental signal that regulates cellular bioenergetics.[1,2] During aerobic fermentation, oxygen plays an important role as the terminal electron acceptor in the respiratory chain that drives the energy metabolism.[3] However, oxygen requirements in aqueous growth medium systems have been estimated to be 14% higher than for conventional aerobic processes. In the case of submerged fermentations, the supply of oxygen to the aqueous phase is one of the limiting factors. Oxygen-vectors are hydrophobic liquids in which oxygen has a higher

solubility than in water.[4] Wang[5] reported the oxygen solubility in n-dodecane was 54.9 mg/L at 35 °C under atmospheric conditions, compared with 34.9 mg/L in water.

Indeed a number of researchers have used oxygen vectors to enhance oxygen solubility successfully,[6] and as a consequence, oxygen vectors have been applied in several different culture systems.[5–7] Examples of common hydrocarbon oxygen vectors include n-dodecane, n-hexadecane, perfluorocarbons and numerous vegetable oils.[8–10] However, it is, not uncommon that overexpressed recombinant proteins in *Escherichia coli* (*E. coli*) show a discrepancy between high specific activity and increased expression level.[11] The expression of protein is a complicated process which is regulated by multiple-signaling pathways, including ones affected by the availability of nutrients, growth factors, and the intracellular microenvironment (ATP, NADH and NADPH).[12,13]

Most studies have concerned the application of oxygen to enhance the yield of target product; however the effects of the intracellular microenvironment (ATP, NADH and NADPH) in the presence of oxygen vector during expression of a fusion protein in *E. coli* have been less extensively studied. Therefore, in the present study, the intracellular microenvironment response to n-dodecane in *E. coli* was investigated, especially the effects on the expression of fumarase.

Materials and methods

Microorganisms, plasmids and growth conditions

Growth conditions and the recombinant *E. coli* BL21-pET22b-fumR, which produces *Rhizopus oryzae* fumarase, have been previously described.[14] The expression of fumarase was induced by adding IPTG (0 h, final concentration 1 mmol/L). Cells were grown for additional 6 h at 30 °C and were harvested by centrifugation.

Oxygen vector

When fumarase expression was induced by adding IPTG, oxygen vector (n-hexane, n-dodecane and n-hexadecane) was immediately added to the sterile fermentation broth at different volumetric fractions as appropriate.

Preparation of cell-free extracts and enzyme assays

Fumarase was purified according to our previously research,[14] and assayed according to the method described by Kanarek and Hill.[15] Protein concentration was determined using the Bradford assay.[16] One unit of fumarase activity was defined as the production of 1 μmol of fumarate or L-malate per minute under standard reaction conditions.

Gel electrophoresis

To determine the amount of soluble fumarase, the supernatant and the pellet fraction after cell disruption were analyzed by SDS-PAGE on a 12.5% separation gel.[17] A Mini-PROTEAN Tear Cell (Bio-RAD, Hercules, CA, USA) was used for electrophoresis.

Table 1 – Effects of different oxygen vectors on fumarase activity.

Oxygen-vectors	Concentration of oxygen-vectors (%, v/v)	OD_{600}	Fumarase activity (U/mg)
Control	0	2.61 ± 0.11	6.44 ± 0.22
n-Hexane	0.6	1.23 ± 0.06	9.32 ± 0.36
n-Dodecane	1.5	2.76 ± 0.12	12.47 ± 0.59
n-Hexadecane	1.5	2.90 ± 0.13	10.01 ± 0.49

Molecular weight markers were purchased from TaKaRa Bio Group. After electrophoresis, the protein bands were visualized with Coomassie Brilliant blue staining.[14]

Intracellular ATP

The intracellular ATP concentration was analyzed by an HPLC system (Dionex P680 pump, Chromeleon controller, and Dionex UVD 170U Detector; Dionex Corporation, CA, USA).[18] One milliliter cell suspension was harvested by centrifugation at $15,000 \times g$ for 1 min at −8 °C and the harvested cells were mixed with 1 mL methanol at −40 °C. A reagent containing 50% perchloric acid was used to extract ATP. The solution was filtered through a 0.22 μm membrane, if necessary, and 20 μL was injected into the HPLC system. The mobile phase comprised phosphate buffer solution/acetonitrile/water (86:4:10) at a flow rate of 1.0 mL/min. The phosphate buffer solution was composed of NaH_2PO_4 10.93 g/L, Na_2HPO_4 3.04 g/L, and tetrabutylammonium bromide 3.22 g/L (pH 6.5). A Sepax HP-C18 column (250 mm × 4.6 mm) was used at 35 °C. The column effluent was monitored at 254 nm.

NAD(P)-NAD(P)H pool isolation and cycling assay

Samples were removed from aerobic cultures, and the dinucleotides were extracted by the method described by Vemuri.[19] Recycling assays of the extracts containing specific dinucleotide species were performed in triplicate.[20]

Results and discussion

Effects of oxygen vectors on fumarase activity

Three oxygen vectors (n-hexane, n-dodecane and n-hexadecane) at a final concentration of 1.5% (v/v) were immediately added to the fermentation broth. The oxygen vectors added in this study were all organic solvents, which have toxicity effects on cells at high concentrations.[21–23] In this study, 1.5% n-hexane was found to be toxic, and was observed to inhibit growth of the cell and cause cell lysis after 3 h. As a consequence, the concentration of n-hexane was decreased to 0.6%. The effects of oxygen vectors on cell growth and fumarase activity were investigated after 6 h of induction (Table 1). The biomass increased after adding 1.5% n-dodecane and n-hexadecane, while it was still inhibited by the 0.6% n-hexane.

Enhancement of fumarase activity was detected. With 1.5% n-dodecane, fumarase activity reached the maximum value of 12.47 U/mg, which increased by 94% compared with con-

Table 2 – Effects of concentrations of *n*-dodecane on fumarase activity.

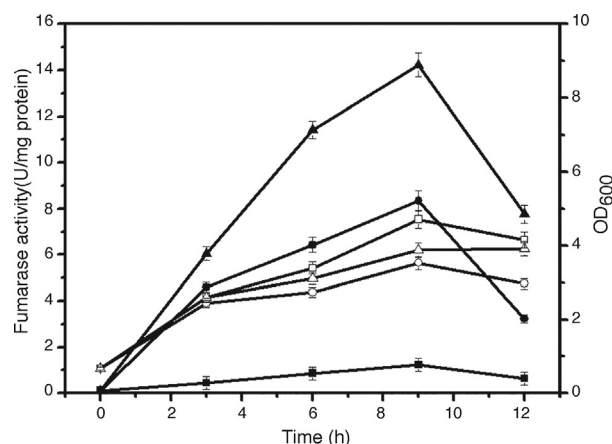| Concentration of *n*-dodecane (%, v/v)	OD_{600}	Fumarase activity (U/mg)
0	2.92 ± 0.15	6.33 ± 0.32
0.5	3.02 ± 0.15	7.09 ± 0.24
1	3.12 ± 0.16	8.96 ± 0.54
1.5	3.21 ± 0.11	10.32 ± 0.52
2	3.07 ± 0.14	12.68 ± 0.59
2.5	3.03 ± 0.14	14.21 ± 0.61
3	2.86 ± 0.13	11.33 ± 0.56
3.5	2.54 ± 0.14	1.32 ± 0.07

Fig. 1 – Effect of 2.5%(v/v) *n*-dodecane on the time-course profiles of fumarase activity. ■, Fumarase activity without IPTG or *n*-dodecane; ●, fumarase activity with IPTG but without *n*-dodecane; ▲, fumarase activity with IPTG and *n*-dodecane; □, OD_{600} without IPTG or *n*-dodecane; ○, OD_{600} with IPTG but without *n*-dodecane; △, OD_{600} with IPTG and *n*-dodecane.

trol experiments. Therefore, this vector was selected for the subsequent investigations.

The concentrations (0–3.5%, v/v) of the *n*-dodecane were added to investigate their influences on cell growth and fumarase activity. Table 2 shows that the addition of *n*-dodecane as an oxygen vector at low concentrations increased biomass production and fumarase activity. At concentration of 2.5% *n*-dodecane, the activity was higher than that observed in the concentrations of 1, 1.5 and 2, despite lower biomass, which may indicate a better folding of the enzyme. However, at volumetric fractions of *n*-dodecane higher than%, cell lysis and decreased fumarase activity were observed, which might have been because higher concentrations of *n*-dodecane are toxic to cells.

Effects of concentrations of 2.5% (v/v) *n*-dodecane on the time-course profiles of fumarase activity

The effect of the presence of 2.5% *n*-dodecane is shown in Fig. 1 illustrates that expression of the *fumR* gene was induced by adding IPTG; meanwhile the biomass was inhibited by IPTG. The OD_{600} and fumarase activity during the induction
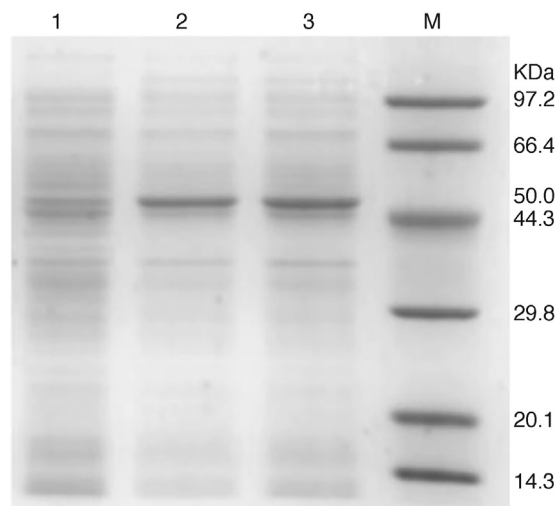

Fig. 2 – Effect of *n*-dodecane on the expression of *fumR* gene. SDS PAGE analysis of the expression levels of the fumR at 37□. The expression of the fumarase was induced by adding IPTG (final concentration 1 mM). Cells were grown for additional 6 h at 180 rpm and 37□ and were harvested by centrifugation. The cells were normalized to their end OD_{600}. M: M: protein marker; 1: pET-22b-fumR,-IPTG, – *n*-dodecane; 2:pET-22b-fumR,+IPTG, – *n*-dodecane:3:pET-22b-fumR,+ – IPTG,+ *n*-dodecane.

process were depicted that the *n*-dodecane keep the activity and biomass at higher level compared with *n*-dodecane absence. After 9 h of induction, the biomass and the fumarase activity both reached the maximum value. The 2.5% *n*-dodecane addition increased fumarase activity to 14.21 U/mg, which is 124% enhancement compared with the absence of *n*-dodecane.

The addition of *n*-dodecane did not induce a significant change in the expression of *fumR* gene (Fig. 2), while the fumarase activity was increased significantly. It is possible that the accurate folding caused the higher activity. Protein folding and expression are complicated processes that are regulated by multiple-signaling pathways including one affected by the availability of nutrients, growth factors, intracellular ATP levels, internal redox state (NADH/NAD),[24] and environmental stress.[12] The addition of an oxygen-vector possibly influenced oxygen transport through the respiratory chain, subsequently changing the intracellular microenvironment.

Changes of intracellular ATP

The universal molecular carrier for biological energy is ATP, and both protein biosynthesis and folding require ATP hydrolysis. The effect of *n*-dodecane and IPTG on ATP concentration are shown in Fig. 3. With the absence of IPTG, the concentration of ATP was continuously rising. While IPTG and *n*-dodecane were both used, the concentration of ATP reached the maximum value at 6 h. And during the first 6 h of induction, the concentration of ATP increased sharply to a value 7700% higher than that obtained without an oxygen-vector, and then it dropped significantly. However, fumarase activity
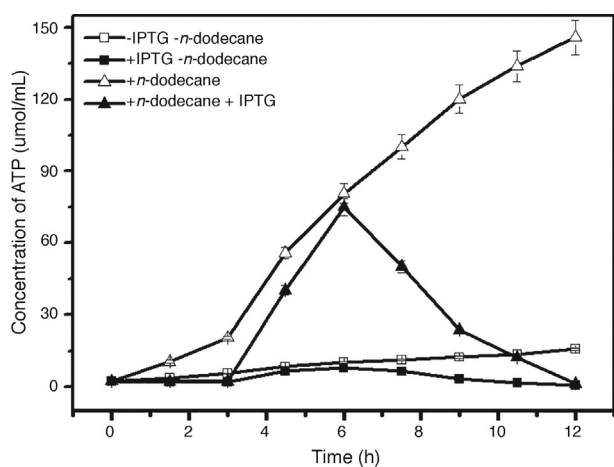
Fig. 3 – The effect of different oxygen vectors on ATP concentration. □,-IPTG -oxygen vector; ■,-IPTG-oxygen vector;△,+ *n*-dodecane; ▲, +*n*-dodecane +IPTG.

(Fig. 1) was increased continuously while the expression level almost kept the same after 6 h induction (Fig. 2). It has been suggested that molecular chaperones actively facilitate protein folding by using the energy of ATP hydrolysis to alter the accessible conformations for a non-native protein.[25,26]

Chaperones are essential for maintaining protein-folding homeostasis and play important roles in the cellular stress response. By binding to aggregation-sensitive folding intermediates, chaperones inhibit aberrant interactions between proteins. The most intensively studied folding chaperones, such as the GroEL–ES and DnaK systems, facilitate substrate protein folding through ATP- and cofactor-driven conformational changes.[25–27] Although a number of ATP-independent chaperones have been reported, most chaperones use cycles of ATP binding and hydrolysis to assist protein folding were well studied.[27,28] The mechanism of nucleotide sensing in group II chaperonins in eukaryotes has been revealed. Nucleotide-sensing loop (NSL) was studied as an ATP binding site. Functional analysis using NSL mutants shows a significant decrease in ATPase activity, suggesting that the NSL is involved in timing of the protein folding cycle. The binding of ATP and subsequent hydrolysis promote the closure of the multi-subunit rings where protein folding occurs.[29]

Changes of intracellular redox state

NAD, NADH, NADP and NADPH are important cofactors involved in biological redox reactions. These cofactors play an important role in preserving and regulating the intercellular redox state. In this study, the concentration changes of those cofactors were investigated after adding IPTG and *n*-dodecane. After adding IPTG, the concentration of NAD and NADH declined (Fig. 4A). During the induction, the concentration of NAD and NAD/NADH ratio gradually increased, reaching the maximum values at 9 h, and subsequently decreased, while the concentration of NADH increased slowly. The addition of oxygen vectors enable the intracellular NAD/NADH ratio increase more significantly. In addition, the same trend occurred in the changes in concentration of NADP, NADPH, and NADP/NADPH ratio (Fig. 4B). NAD/NADH and NADP/NADPH ratios were positively correlated with fumarase activity.

The proper folding and stability of many proteins depend on the formation of disulfide bonds.[30] The higher NAD/NADH and NADP/NADPH ratios are more suitable for the formation of disulfide bonds, resulting in proper protein folding. Protein Data Bank (PDB) contains several fumarase crystal structures, although none of them has disulfide bonds. *R. oryzae* fumarase presents 6 cysteines residues, being possible the formation of disulfide bonds, differently from the others fumarase. Hence, one possibility is that the additional NADH oxidation occurs through electron transport chain (ETC) and more ATP is produced to provide enough energy for the formation of disulfide
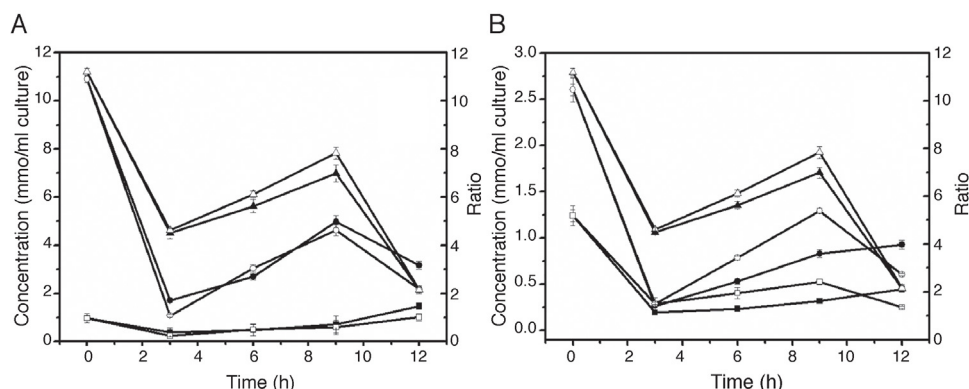

Fig. 4 – Effect of *n*-dodecane on intracellular redox state. A: Effect of *n*-dodecane on the NAD/NADH ratio. ■, the concentration of intracellular NADH without adding *n*-dodecane; ●, the concentration of intracellular NAD without adding *n*-dodecane; ▲,NAD/NADH ratio without adding *n*-dodecane; □, the concentration of intracellular NADH adding with *n*-dodecane; ○, the concentration of intracellular NAD adding with *n*-dodecane; △,NAD/NADH ratio adding with *n*-dodecane. B: Effect of *n*-dodecane on the NADP/NADPH ratio. ■, the concentration of intracellular NADPH without adding *n*-dodecane; ●, the concentration of intracellular NADP without adding *n*-dodecane; ▲,NADP/NADPH ratio without adding *n*-dodecane; □, the concentration of intracellular NADPH adding with *n*-dodecane; ○, the concentration of intracellular NADP adding with *n*-dodecane; △,NADP/NADPH ratio adding with *n*-dodecane.

bonds, and for protein folding. NADPH can also enter the mitochondrial ETC through complex reactions. With the presence of n-dodecane, the over-express fumarase from *R. oryzae* by recombinant *E. coli* causes cells to produce more ATP for the protein expressing and folding, requiring more energy from NADH oxidation through the respiratory chain.

Conclusions

The effect of oxygen-vectors disturbance on the intracellular microenvironment of the submerge fermentation of fumarase overexpression in *E. coli* was investigated. In this study, the addition of n-dodecane increased fumarase activity and the concentration of ATP. Redox ratios significantly increased, while a significant change in the expression of the *fumR* gene was not induced (Fig. 4). Thus, on the basis of the above phenomenon, it may be inferred that the addition of an oxygen-vector changed the intracellular energy metabolism. A more oxidative environment can facilitate the correct folding of a protein that present disulfide in the surface. This fumarase possess 6 Cys that could be involved in structural disulfide formation.

In conclusion, application of n-dodecane as an oxygen vector can greatly enhance the expression of fumarase in recombinant *E. coli*, and the biological energy molecule ATP may play a key role in protein expression for both high expression levels and specific activities, which suggests a new strategy in the heterologous expression of protein.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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