Hydrogen peroxide production using chemically treated *Pichia pastoris* cells

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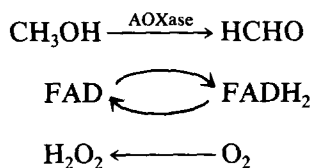
A new method has been developed to prepare intact cell systems to produce hydrogen peroxide (H_2O_2) using methylotrophic yeast Pichia pastoris. *The high alcohol oxidase (EC 1.1.3.13; AOXase) content in the living* P. pastoris *cells is always associated with high activity of catalase (about 200- to 400-fold higher than that of AOXase) to protect the cells from oxidative damage from H_2O_2. To obtain catalase-free intact cells, a combination of a cationic detergent, cetyldimethylbenzyl-ammonium chloride (Cation M2), to permeabilize the cell membrane and a catalase inhibitor, sodium azide, was used to selectively release and inactivate the cellular catalase. This chemical treatment released more than 50% of the cellular protein including the inactivated catalase, thus increasing more than twofold the specific cellular AOXase activity. In addition, the cells were made more permeable to the reacting substrates. Using chemically treated* P. pastoris *YB4290 cells, 10 mM of H_2O_2 was produced from 10 mM methanol (Tris-HCl buffer, pH 7.5) in 4 h at 15°C. In the presence of pure oxygen instead of air, 50–80 mM of H_2O_2 was produced in 3 h at a maximum rate of 2.6 M g^{-1} (dry cell wt.) h^{-1}. The cells maintained half of the initial enzyme activity after five repeated-batch experiments at 15°C.*

Keywords: *Pichia pastoris;* alcohol oxidase; catalase; hydrogen peroxide; production; treated cells

Introduction

Methylotrophic yeasts of genera such as *Pichia, Hansenula*, and *Candida* synthesize alcohol oxidase (AOXase), which allows them to use methanol as their sole source of carbon and energy. In the presence of oxygen, AOXase catalyzes the oxidation of methanol to formaldehyde and generates a coproduct, hydrogen peroxide, as the first catalytic step of the methanol dissimilatory pathway.[1]



In methanol medium, AOXase can be induced to more than 30% of the total cellular protein.[2,3] AOXase has broad substrate specificity and can oxidize a variety of short-chain primary alcohols other than methanol.[1,2,4]

Due to its unique properties, methylotrophic yeast AOXase has been applied to various areas of biotechnology. For instance, AOXase has been used as an alcohol scavenger[5] and as part of a recovery system for dilute ethanol streams.[6] As a catalyst for the production of H_2O_2, AOXase has been used in colorimetric determination of alcohols, for bleaching processes, as an antimicrobial system, and for the degradation of toxic organic compounds.[7,8] Since many applications of AOXase rely on the production or detection of H_2O_2, it is evident that removal of catalase from cellular extracts of methanol-grown yeasts is an important step in the production of this enzyme. The main constraint on using the purified AOXase has been the cost of the purified enzyme.[7]

Whole, methanol-grown yeast cells containing AOXase have been used as biocatalysts in the production of formaldehyde[9] and flavor aldehydes.[10] In general, whole-cell systems are more economical than using a pure enzyme system. However, due to the presence of catalase in the whole cell, this system cannot be used directly to produce H_2O_2. One option is to use a genetically modified catalase-negative strain. However, it has been shown that the maximum productivity of AOXase by a genetically modified catalase-negative strain *Hansenula polymorpha* is only 50% of that in the wild-type strain.[3] Also, this mutant still decomposed H_2O_2 through another mechanism.[11]

One alternate approach is the use of chemical treat-

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ment to achieve catalase-free whole cell systems with high AOXase activity that can produce H_2O_2. Chemical permeabilization of microorganisms has been demonstrated to selectively release intracellular proteins based on intracellular location by altering the cell envelope structure without lysing the cell.[12] Potentially, chemical permeabilization can be used to selectively remove intracellular proteins based on molecullar weight. Since catalase (MW 240,000) is relatively smaller than AOXase (MW 600,000),[13] catalase may be selectively released from the cell while keeping the AOXase inside the cell. Besides catalase, other proteins such as membrane proteins and proteases may also be removed to increase the permeability of the cell wall, to increase the specific activity, and to stabilize the cellular AOXase. Such naturally "immobilized" cellular biocatalysts may provide promising economic alternatives to purified AOXase for various applications. We demonstrate here a successful preparation of cellular biocatalysts to produce H_2O_2 using *Pichia pastoris* cells by a combination of chemical permeabilization and catalase inactivation.

Materials and methods

Culture and conditions

P. pastoris NRRL Y11430 and YB4290 (Northern Regional Research Center, Peoria, IL) were grown on modified yeast medium (MYM) at 30°C with constant shaking for preculture. MYM contains, per liter distilled water: 3 g yeast extract, 3 g malt extract, 5 g peptone, and 20 g cerelose. The cultures were inoculated with 1% (v/v) into methanol medium (MM) and allowed to grow to late exponential phase at 30°C. MM contains, per liter distilled water: 6.75 g yeast nitrogen bases without amino acids, 12 g KH_2PO_4, and 2.1 g K_2HPO_4. The pH was adjusted to 6.0 prior to autoclaving, and 1% (v/v) methanol was aseptically added immediately prior to inoculation. Cell growth was monitored by turbidity, measured using a spectrophotometer DMS90 (Varian, Palo Alto, CA) at 600 nm. The cells were recovered by centrifuging (3,000*g*, 10 min) and washed twice in cold treatment buffer [0.1 M potassium phosphate buffer (KPB), pH 7.0].

Chemical permeabilization

Cells were resuspended in a 2-l flask with the treatment buffer to give a final concentration of 1.5 g (dry cell wt) l^{-1} with a typical working volume of 1 l. The solutions of 1% (w/v) cetyldimethylbenzyl-ammonium chloride (Cation M2) and/or 500 mM sodium azide in the same buffer were added to the cell suspensions to yield the indicated concentrations of treatment chemicals. The flasks were left gently shaking at 30°C. The samples were withdrawn at the indicated intervals and washed twice with the same buffer before assay for their AOXase and catalase activities. Treated cells were collected by centrifuging and washed three times with the buffer. The cells were resuspended in cold deionized water and freeze-dried. The freeze-dried cells were stored at −20°C.

Assays

The AOXase activity was determined by following the formation of H_2O_2 by the 2,2′-azide-bis(3-ethyl)-benzthiazoline-6-sulfonic acid (ABTS)-peroxidase method as described elsewhere.[14] The ABTS reagent contained 16 mg of ABTS, 1 mg of horseradish peroxidase, and 2 ml of methanol in 98 ml 0.1 M KPB (pH 7.0). The assay mixture was prepared with 2.5 ml of the ABTS reagent and 25 μl of the AOXase sample (cellular extract or whole cell suspension) solutions in a 4 × 1 × 1 cm cuvette and maintained at 23°C. The increase in absorbance at 390 nm was recorded for 2 to 4 min. One unit (U) of AOXase was defined as the amount of enzyme that formed 1 μmol of H_2O_2 per minute under the conditions of assay. An extinction coefficient of 43,570 M^{-1} cm^{-1} at 390 nm was used. Catalase was assayed spectrophotometrically according to Bergmeyer.[15] The incubation mixture contained 0.1 M KPB (pH 7.0) and 16.5 mM H_2O_2. The decrease in absorbance at 240 nm was monitored at 23°C. A molar extinction coefficient of 40.67 M^{-1} cm^{-1} at 240 nm was used for H_2O_2. One enzyme unit of catalase activity was defined as the amount of enzyme that decomposed 1 μmol of H_2O_2 per minute under the conditions of assay. H_2O_2 was assayed by the ABTS-peroxidase method. Methanol was omitted in the ABTS reagent for measurement of H_2O_2. Formaldehyde was assayed according to the method of Nash.[16] In most cases, three measurements were carried out for each assay. All values represent the average values of these measurements. Measurement errors were within 5%.

Preparation of cell-free extracts

The cell-free extracts from chemically treated and nontreated cells were prepared by sonication using a Sonic Dismembrator Model 300 (Fisher, Pittsburgh, PA). The cell suspension of 1.5 g (dry cell wt.) l^{-1} in 0.1 M KPB (pH 7.0) was treated 20 times for 30 s at 30-s intervals at 0°C. Cell debris was eliminated by a 15-min centrifugation at 12,000*g* and the resulting supernatant was diluted to the appropriate concentration for protein analysis or other applications.

Protein analysis

Protein was determined with the BCA protein assay kit obtained from Pierce (Rockford, IL) using bovine serum albumin as standard. The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a PhastSystem separation and development unit (Pharmacia, Uppsala, Sweden). Phastgel homogeneous 12.5% medium was used. Protein bands were visualized by silver staining.[17] Low-molecular-weight marker proteins were also from Pharmacia.

Production of H_2O_2

A typical reaction mixture consisted of a volume of 2.5 ml containing 10 g (dry cell wt.) l^{-1} of chemically treated cells and 10 mM methanol in 0.1 M Tris-HCl buffer (pH 7.5) in a 1.4 × 13 cm glass reactor maintained at tested temperatures through a water bath jacket with stirring in an air-saturation condition. The air in the reactor was replaced by pure oxygen when it was necessary, and then the reactor was sealed with a rubber stopper. Oxygen was occasionally supplied to the reactor during the reaction. Samples (10 μl) were withdrawn at the indicated intervals into a microtube with proper dilution and centrifuged at 12,000*g* for 1 min. In this way, the cells were removed to stop the reaction.

Materials

Cetyldimethylbenzyl-ammonium chloride (Cation M2) was purchased from Fluka Chemika-BioChemika (Buchs, Swit-

Table 1 Comparison of chemical treatment methods of *P. pastoris* cells using different detergents

Chemical reagents	Detergent type	Treatment conditions	Cellular protein released	AOXase released	Reference
0.1% Triton X-100 + 1% glycine	Non-ionic	30°C, 24 h	50%	No	14
1% SDS + 2% xylene	Anionic	25°C, 2 days	-	Yes	18
0.1% Cation M2	Cationic	30°C, 2 h	47%	No	This work

zerland). 3-Amino-1,2,4-triazole was obtained from Sigma Chemical (St. Louis, MO). The sources of other chemicals have been described elsewhere.[14]

Results and discussion

Chemical permeabilization of P. pastoris *cells*

Cation M2 was used to permeabilize *P. pastoris* Y11430 cells. Cation M2 was added to cell suspensions to final concentrations of 0.1 and 0.3% (w/v). The treatment was carried out at 30°C with gentle shaking to keep the cells suspended. AOXase and catalase activities were measured in both the supernatant and the washed intact cells. Catalase was detected in both the supernatant and the intact cells during the treatment. It was found that more than 80% of catalase was removed from the cells by treatment with 0.1% Cation M2 for 3 h. AOXase activity was detected only in the washed intact cells. This suggested that this treatment can retain AOXase inside the cell. However, the AOXase activity of the washed cells decreased to one-third of its original activity in a 3-h treatment. The loss of AOXase activity was more severe when higher concentrations (>0.3%) of Cation M2 were used. The permeabilization efficiency in terms of total cellular protein release using Cation M2 alone was similar to that using Triton X-100 plus glycine,[14] but the treatment time was reduced. In both cases, AOXase was retained inside the cell. *Table 1* summarizes the various chemical treatments of methylotrophic *P. pastoris* cells using different detergents. Cation M2 is superior to the other detergents in terms of treatment time and enzyme retention within the cells. The anionic detergent sodium dodecyl sulfate (SDS) has been known to selectively destroy the catalase activity,[7] but it also releases the AOXase from the cell.[18] Detergent-treated cells appear physically similar to the original cell and form hard pellets after centrifugation. Although additional amounts of catalase can be removed using longer periods of treatment, AOXase activity is also observed to decrease. An additional inactivation method is needed to completely eliminate the residual catalase activity.

Catalase inactivation and stabilization of the P. pastoris *cells*

Inhibitor-based inactivation was used to further eliminate residual catalase activity of these cells. Two catalase inhibitors, 3-amino-1,2,4-triazole[19] and sodium azide,[20] were compared for effectiveness of catalase inactivation and effects on AOXase. Different amounts of aminotriazole and sodium azide were mixed with cell extracts from *P. pastoris* Y11430, which contains both catalase and AOXase. The mixtures were incubated at room temperature for 10 min. The remaining activities of both catalase and AOXase were measured. Although catalase can consume H_2O_2, its presence does not interfere with the ABTS-peroxidase assay for AOXase.[14] This was tested by adding catalase to AOXase solution with a ratio of 1,000 : 1 in enzyme units and then measuring the AOXase activity of the mixture. The activities of AOXase in these enzyme mixtures were found to be similar to that of pure AOXase using the ABTS-peroxidase assay. As shown in *Table 2*, 5 mM sodium azide inactivated more than 99% of the catalase (CATase) activity. It is a much stronger inhibitor than aminotriazole, which can only inactivate 90% of catalase activity at a concentration of 500 mM. A 5 mM sodium azide concentration does not seem to have any effect on the AOXase activity of the cell extracts, but a 10 mM concentration of sodium azide strongly inhibited the AOXase activity. Aminotriazole reduced the AOXase activity by 9 to 15% at both 100 and 500 mM concentrations. These results demonstrate that sodium azide is a better reagent to eliminate residual catalase activity in this system. Although sodium azide has been reported to be a competitive inhibitor of AOXase,[1,4] lower concentrations of sodium azide (<5 mM) do not seem to have any effect on cellular AOXase activity and can selectively inactivate the more sensitive catalase. Preliminary experiments showed that 5 mM sodium azide in a cell suspension of 1.5 g (dry cell wt.) l^{-1} is a suitable concentration to completely inactivate catalase without reducing AOX-

Table 2 Treatment of cell-free extracts from *P. pastoris* Y11430 by catalase inhibitors

Catalase inhibitor	Remaining activity (%)				
	Control	Aminotriazole (mM)		Sodium azide (mM)	
		100	500	5.0	10
CATase	100	13	10	0.4	0.2
AOXase	100	91	85	107	51

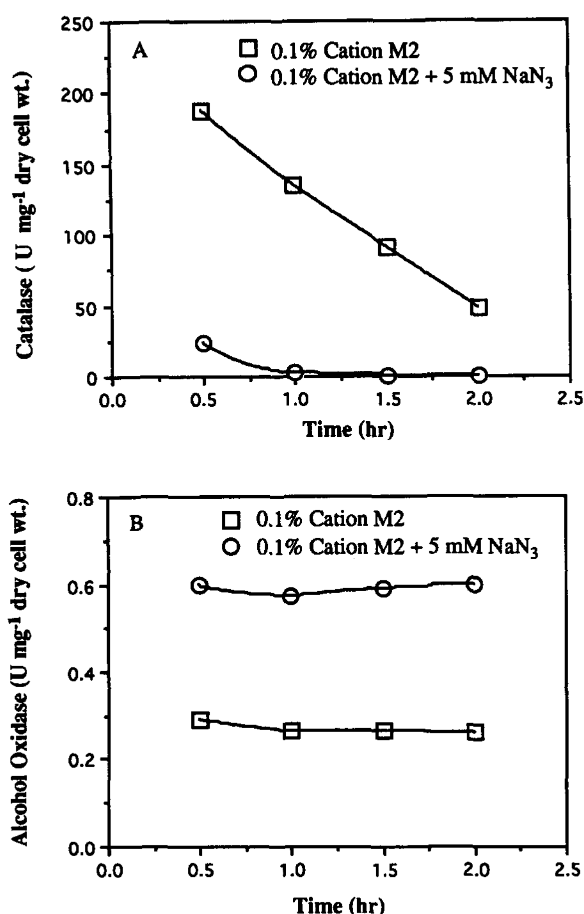

Figure 1 Profile of the chemical treatment of *P. pastoris* Y11430 cells using 0.1% Cation M2 only and 0.1% Cation M2 plus 5 mM NaN_3 at 30°C. (A) Catalase activity of the intact cells. (B) AOXase activity of the intact cells

ase activity. The color of the cells changed from yellow to red as soon as sodium azide was added. This is believed to occur due to the binding of azide to AOXase. Azide has been shown to bind AOXase to form a red enzyme-azide complex which has catalytic properties indistinguishable from the native yellow form of the enzyme.[1,4] Azide was also shown to be able to dissociate from the complex in the presence of substrate to give a fully functional AOXase. Furthermore, Sakai and Tani[4] showed that the presence of azide improved the stability of AOXase of *C. boidinii* S2 against products such as H_2O_2 and aldehydes. Binding of azide to AOXase might also protect the enzyme from further detergent inactivation (data not shown). Therefore, sodium azide was used together with the detergent Cation M2 for chemical treatment of cells.

As shown in *Figure 1A*, when sodium azide was added to a cell suspension with a final concentration of 5 mM together with 0.1% Cation M2 at 30°C, the catalase activity of these cells decreased immediately. After 2 h, catalase activity could not be detected. AOXase activity of the cells dropped immediately to one third of its original level using Cation M2 alone. However, AOXase activity remained at its original high level when the cells were treated with sodium azide and Cation M2 together (*Figure 1B*). This clearly demonstrates that sodium azide is not only inactivating the catalase activity but may also be playing the essential role of stabilizing the AOXase activity.

A similar combination of Cation M2 and sodium azide treatment was applied to another strain of *P. pastoris* YB4290. The results are summarized in *Table 3*. The enzyme activities were always negligible in the untreated intact cells. The treated intact cells show higher AOXase activities compared to the untreated intact cells, especially when sodium azide was used in the treatment. Over 80% of total cellular AOXase activities can now be measured in the treated intact cells. This is primarily due to high initial enzyme concentration inside the cell and a decrease in the apparent K_m values (data not shown), which suggests alteration of the mass transfer resistance of the cell envelope and/or of the peroxisomal membrane. The treated cells retained 86 and 100% of the original AOXase activities for YB4290 and Y11430, respectively. YB4290 cells tend to form aggregates during the treatment. Some of them were observed to adhere to the flask surface. The loss of these cells may also be one factor causing significant loss of the total AOXase activity of YB4290 cells. Most importantly, this chemical treatment can completely inactivate the catalase activity within a period of 2 h. Chemical permeabilization by Cation M2 removed 47 and 63% of the cellular proteins for Y11430 and YB4290 cells, respectively. Higher permeabilization efficiency for YB4290 cells was observed. The specific AOXase activity of treated cells increased more than twofold and was about half of those of purified enzymes (10–12 U mg^{-1}).[1,18] By using a combination of Cation M2 and sodium azide, catalase-free high AOXase activity of whole cells from both strains was obtained. The results of SDS-PAGE of cell-free extracts from treated cells clearly show that AOXase is retained inside the cell after chemical treatment (*Figure 2*, lanes 3 and 5). The background, cellular proteins are "cleaned up" by this treatment, although a few proteins may still exist within the cell. Higher permeabilization efficiency of YB4290 cells compared to Y11430 cells can also be confirmed, since fewer background proteins were detected in YB4290 cell extracts.

The treated cells were obtained either in cell suspension or in dry powder form through freeze-drying. The recovery of the AOXase activity during freeze-drying process was about 65–75%. Higher recovery rates could be expected through addition of stabilizers and optimization of the process. The cell suspension was stored in 10 mM KPB (pH 7.0) at 4°C for a week without any loss of activity. The dry powder was stored at −20°C for 2 months with 10% loss of the activity.

Production of H_2O_2 by the chemically treated P. pastoris *cells*

Figure 3 shows that H_2O_2 could be produced by chemically treated Y11430 and YB4290 cells when methanol

Table 3 Comparison of the activities of AOXase and catalase of *P. pastoris* Y11430 and YB4290 cells before and after chemical permeabilization and/or catalase inactivation at 30°C for 2 h. All values represent the average values from two different batches of cells

		Activity ($U mg^{-1}$ dry cell wt)				Specific activity ($U mg^{-1}$ cellular protein)				
		Intact cell		After sonication		Intact cell		After sonication		Cellular protein ($mg mg^{-1}$ dry cell wt)
P. pastoris	Treatment	AOXase	CATase	AOXase	CATase	AOXase	CATase	AOXase	CATase	
	Control	0.01	3	0.71	270	0.02	8	2.22	840	0.32
Y11430	0.1% Cation M2	0.25	48	0.30	56	1.67	320	2.00	373	0.15
	0.1% Cation M2+ 5 mM NaN_3	0.60	0	0.72	0	4.00	0	4.80	0	0.15
	Control	0.01	2	1.45	291	0.03	5	3.82	765	0.38
YB4290	0.1% Cation M2	0.25	14	0.27	15	1.92	108	2.08	115	0.13
	0.1% Cation M2+ 5 mM NaN_3	0.93	0	1.25	0	6.64	0	8.93	0	0.14

was used as a substrate, whereas untreated cells were unable to produce H_2O_2 in any measurable quantities. Although we observed a higher initial H_2O_2 production rate at 23°C than that at 15°C (in the first few minutes), the production rate decreased rapidly at 23°C resulting in lower final H_2O_2 concentration. This result suggests that the enzyme was significantly inactivated at the higher temperature. Using the same cell concentration, YB4290 cells produced more H_2O_2 than Y11430 cells. This reflects the higher AOXase specific activity of YB4290 cells. The other product of the reaction, formaldehyde, was also detected in stoichiometric quantities. Higher concentrations of H_2O_2 were produced us-

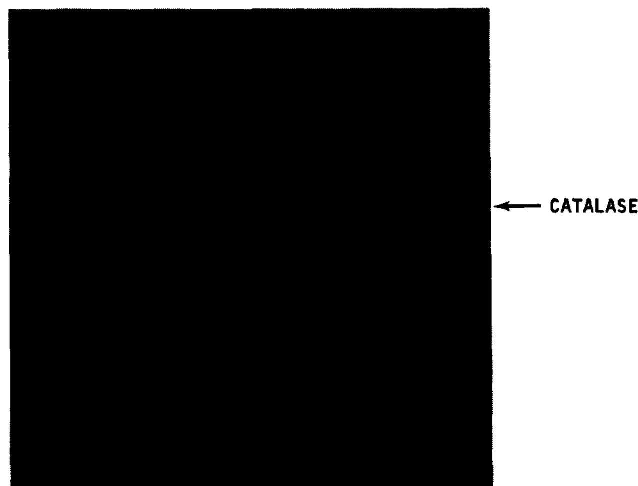

Figure 2 SDS-PAGE of the cell-free extracts from *P. pastoris* Y11430 and YB4290 cells. Lane 1, purified AOXase (Sigma Chemical); lane 2, extracts from *P. pastoris* Y11430 cells; lane 3, extracts from *P. pastoris* Y11430 cells treated with 0.1% Cation M2 and 5 mM NaN_3 for 2 h at 30°C; lane 4, extracts from *P. pastoris* YB4290; lane 5, extracts from *P. pastoris* YB4290 cells treated with 0.1% Cation M2 and 5 mM NaN_3 for 2 h at 30°C. Cell-free extracts were prepared from the same concentration of cell suspension solution for each strain

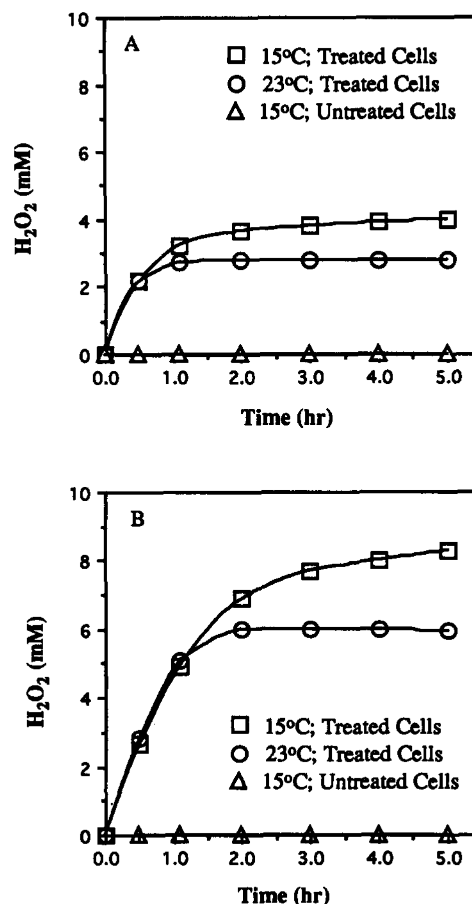

Figure 3 Production of H_2O_2 by 0.1% Cation M2 plus 5 mM NaN_3-treated and NaN_3-untreated cells at 15 and 23°C. (A) *P. pastoris* Y11430; (B) *P. pastoris* YB4290. Reaction conditions: 10 g dry cell wt l^{-1}; 10 mM methanol; air-saturated batch reactor (2.5 ml); 0.1 M KPB (pH 7.0)

Table 4 Effect of H_2O_2 and HCHO on AOXase stability of the treated *P. pastoris* cells at 23°C

	$t_{1/2}$ (h)					
	0.1 M KPB (pH 7.0)			0.1 M Tris-HCl (pH 7.0)		
P. pastoris	Control	10 mM H_2O_2	10 mM HCHO	Control	10 mM H_2O_2	10 mM HCHO
Y11430	0.55 ± 0.04	0.27 ± 0.02	0.08 ± 0.01	1.20 ± 0.11	0.68 ± 0.05	1.00 ± 0.08
YB4290	2.50 ± 0.25	0.57 ± 0.06	0.16 ± 0.01	5.10 ± 0.42	1.30 ± 0.42	2.00 ± 0.11

ing both cells when Tris buffer was used instead of phosphate buffer at pH 7.0. The cellular AOXase of both Y11430 and YB4290 cells are more stable in Tris buffer than in phosphate buffer (*Table 4*). The H_2O_2 concentrations produced by the cell are comparable with the concentrations produced by purified enzyme.[7] This system provides a relatively economic way for *in situ* H_2O_2 generation. The H_2O_2 concentration obtained in this system is sufficient for bleaching purposes, to kill microorganisms,[7] and to oxidize various toxic organic compounds.[8]

Figure 4 shows the effect of initial methanol concentration on H_2O_2 production by the treated YB4290 cells. The conversion rate was observed to be 100% at lower methanol concentrations (<10 mM). The reaction was complete in 2 to 4 h. At a concentration of 25 mM methanol, about 92% of the substrates were converted to products. However, at concentrations of methanol 50 mM and higher, low conversion rates were obtained because of significant enzyme inactivation by the products. Substrate stabilization of the cellular AOXase caused higher H_2O_2 concentration (36 mM) using a substrate concentration of 100 mM methanol when compared to only 30 mM of H_2O_2 obtained at 50 mM methanol concentration.

The H_2O_2 production was shown to be oxygen transfer-limited. As shown in *Figure 5*, the H_2O_2 production rate could be increased when pure oxygen was used rather than air-saturated solution. The maximum specific H_2O_2 production rate using pure oxygen was obtained to be 2.6 M g^{-1} (dry cell wt) h^{-1}, and a final concentration of 50 mM H_2O_2 could be produced in 3 h. By adding more cells and substrate, up to 80 mM of H_2O_2 could be produced.

Stability of the treated cells

Hydrogen peroxide and formaldehyde were incubated with both treated Y11430 and YB4290 cells to test the effects of the products on the stability of AOXase of the cells. It was found that the AOXase of both treated cells was inactivated by products in the phosphate buffer (pH 7.0) (*Tables 4* and *5*). Formaldehyde was a much stronger inhibitor/inactivator than H_2O_2. In Tris buffer, AOXase inactivation by formaldehyde was

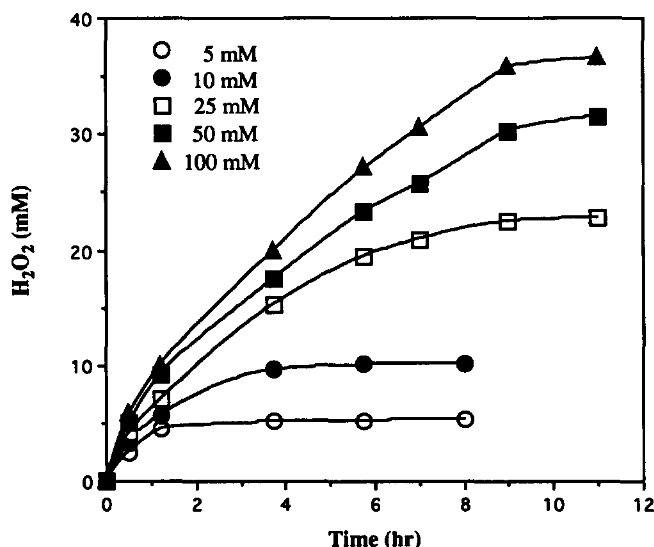

Figure 4 Effect of initial methanol concentration on H_2O_2 production by the treated *P. pastoris* YB4290 cells. Reaction conditions: 10 g dry cell wt l^{-1}; 0.1 m Tris-HCl (pH 7.5); air-saturated batch reactor (2.5 ml); 15°C

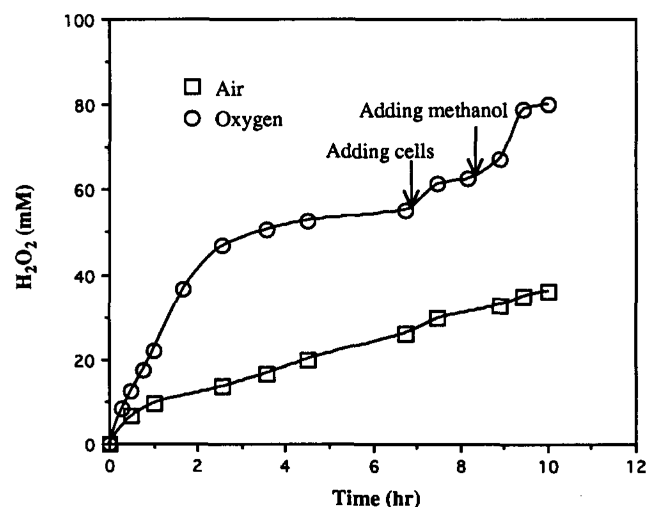

Figure 5 Effect of oxygen on H_2O_2 production by the treated *P. pastoris* YB4290 cells. Reaction conditions: 10 g dry cell wt l^{-1}; 100 mM methanol; 0.5 m Tris-HCl (pH 7.5); oxygen-saturated batch reactor (2.5 ml); 15°C

Table 5 Effect of H_2O_2 and HCHO in 0.1 M Tris-HCl (pH 7.0) on AOXase stability of the treated *P. pastoris* cells at 15°C

	$t_{1/2}$ (h)			
P. pastoris	Control	10 mM H_2O_2	10 mM HCHO	10 mM H_2O_2 + 10 mM HCHO
Y11430	12.0 ± 0.88	4.50 ± 0.32	6.80 ± 0.45	-
YB4290	45.0 ± 1.80	8.70 ± 0.75	11.0 ± 0.86	2.50 ± 0.26

Table 6 Effect of dithiothreitol (DTT) and phenylmethylsulfonyl fluoride (PMSF) in 0.1 M Tris-HCl (pH 7.0) on AOXase stability of the treated *P. pastoris* cells at 23°C

	$t_{1/2}$ (h)		
P. pastoris	Control	1 mM DTT	1 mM PMSF
Y11430	1.20 ± 0.11	7.10 ± 0.22	1.90 ± 0.12
YB4290	5.10 ± 0.42	26.0 ± 0.99	7.20 ± 0.52

greatly reduced. This result demonstrates that there is an interaction between Tris buffer and formaldehyde that decreases the amount of free formaldehyde in the aqueous phase. *Table 5* shows that the AOXase of both treated cells was relatively stable at 15°C. Inactivation of AOXase activity by H_2O_2 and formaldehyde was shown to be synergistic. As shown in *Table 6*, the AOXase activity was greatly improved in the presence of 1 mM dithiothreitol (DTT). Some protease-induced degradation was also observed.

The stability of AOXase is a key factor to enhance H_2O_2 production. Both products, H_2O_2 and formaldehyde, can inactivate/inhibit the enzyme. The stability of the enzyme can be enhanced if the products are removed periodically. Several repeated-batch experiments for H_2O_2 production using treated YB4290 cells were carried out. The results are shown in *Figure 6*. The cells lost half the enzyme activity in two batches (200 min) at 23°C. However, the cells maintained half of the original enzyme activity in 5 batches (500 min) at 15°C.

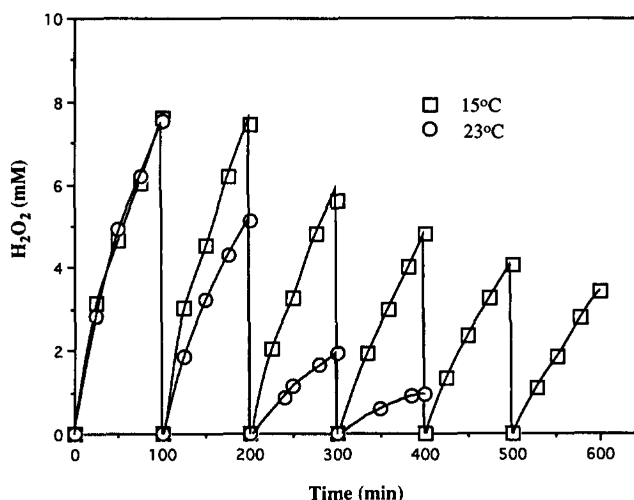

Figure 6 Repeated-batch experiments of H_2O_2 production by the treated *P. pastoris* YB4290 cells at 15 and 23°C. Reaction conditions: 10 g dry cell wt l^{-1}; 10 mM methanol; air-saturated batch reactor (2.5 ml); 0.1 m Tris-HCl buffer (pH 7.5). After each batch reaction (every 100 min), the cells were centrifuged and washed twice with the same buffer at 4°C

Conclusions

Naturally "immobilized" cellular biocatalysts with high AOXase activity were developed by chemical treatment of *P. pastoris* cells for H_2O_2 production. Specific AOXase activities were enhanced twofold through chemical permeabilization. The maximum H_2O_2 concentrations produced by the cell systems were as high as 50–80 mM. These intact cell systems provide a relatively economic method for *in situ* H_2O_2 generation. The H_2O_2 concentration obtained in this system is sufficient for bleaching, for killing microorganisms, and for oxidizing various toxic organic compounds.

Acknowledgements

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