

MICROBIAL HALOGENATION WITH SPECIFIC REFERENCE TO CHLOROPEROXIDASE*

D.C. SMITH

A.T. BULL

G. HOLT

The Biological Laboratory,
The University of Kent at Canterbury,
Kent, CT2 7NJ England

C. CHRISTY

M.D. LILLY

Department of Chemical and Biochemical Engineering,
University College London,
London WC1E 7JE, England

C. RICE

The School of Biotechnology,
The Polytechnic of Central London,
London W1M 8JS, England

ABSTRACT

Chloroperoxidase (EC.1.11.1.10) is produced from the fungus *Caldariomyces fumago*. Mutational and physiological optimisation strategies have been employed to overcome problems of using a wild-type strain concerned with low growth rate, excretion of large amounts of a melanin-type pigment, pelleting of the mycelia, lowering productivity and interference in the ultrafiltration step of downstream processing. Mutant CF 506 was successfully used in a scaled up process to 1000 litres. The occurrence of halogenating enzymes in nature is broad and a wide spectrum of reactions can be catalysed. In order to screen microorganisms for novel haloperoxidases a plate test using commercially-available dyes to enable chloroperoxidase (CPO) producers to be excluded has been devised. Compounds belonging to the arylmethane dyes particularly triphenols such as xylene cyanol (C.I. No. 43535) were decolourized by chloroperoxidase. Organisms able to incorporate the radiohalide Cl^{36} but giving a negative CPO test are now under study.

* Paper presented by Professor G. Holt at the UNESCO Regional Workshop, "The Exploitation of Biotechnology Techniques in the Production and Utilization of Extra-Cellular Enzymes", Bangkok, Thailand, 24-28 March 1986.

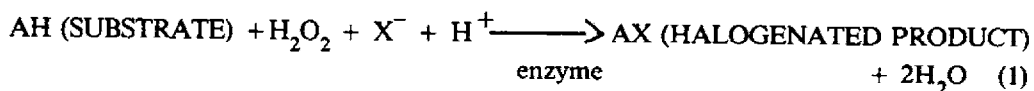
INTRODUCTION

1 Halometabolites

The first halometabolite 3, 5-Diiodotyrosine was reported in 1896 by Drechsel as isolated from the coral *Gorgonia cavolinii*. In 1986 more than 800 halometabolites are known. Examples of fluorine - chlorine - bromine - and iodine - metabolites can be found throughout the living kingdom. Table 1 shows the distribution reported so far.

2 Haloperoxidases

These are enzymes that can catalyse the peroxidative formation of carbon-halogen bond in the presence of hydrogen peroxide, halide ion and a suitable halogen acceptor. The enzymic halogenation reaction can be illustrated as



The haloperoxidases are classified according to oxidation-reduction potentials and hence to the range of halide ions (X^-) that can be oxidised; chloroperoxidase can utilize Cl^- , Br^- and I^- , bromoperoxidase, Br^- , I^- and iodoperoxidase only I^- . Haloperoxidases using H_2O_2 cannot oxidize fluorine ions.

Most nucleophiles with an activated position available for attack by an electrophilic halogen species appear to serve as a suitable halogen acceptor, AH, in this reaction. The mechanism of the peroxidative halogenation reaction is still in dispute. There is both evidence for the intermediary formation of an enzyme bonded electrophilic halogenated intermediate¹ as well as a free radical intermediate mechanism.²

TABLE 1 DISTRIBUTION OF HALOMETABOLITES IN NATURE

Identified Metabolite Containing	HUMANS	HIGHER ANIMALS	HIGHER PLANTS	ALGAE	FUNGI	BACTERIA
IODINE	+	+	-	+	-	-
BROMINE	-	+	+	+	-	+
CHLORINE	-	+	+	+	+	+
FLUORINE	-	-	+	-	-	+

From studying halogenation in our laboratories and from the literature it is evident that the variety of halogenation enzymes is large. The occurrence of halogenating enzymes in nature is broad and a wide spectrum of reactions can be catalysed. No enzymes have been isolated to date that directly activates F^- . The specificity of these haloperoxidases lies in their ability to utilize a particular halogen ion. For example, thyroid peroxidase is able to iodinate independently of interference from the more prevalent Cl^- . The chlorinating enzymes have been selected to take advantage of the most common halide ion Cl^- in the presence of the more reactive Br^- and I^- ions. The enzymes that activate Br^- and I^- but not Cl^- offer a unique opportunity to synthesize heterogeneous dihalide compounds.³

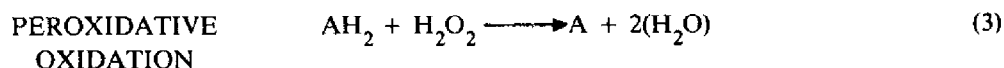
CHLOROPEROXIDASE (CPO)

1 Introduction

Chloroperoxidase (E.C. 1.11.1.10) was discovered by Raistrick's group in 1940.⁴ It is an extra-cellular haem glycoprotein produced by the imperfect fungus *Leptoxyphium* (= *Caldariomyces*) *fumago* from which it has been crystallized and characterized.⁵ It contains one ferriprotophorphyrin IX prosthetic group per enzyme molecule and is a glycoprotein (mol. wt. 42,000) of which 25% is carbohydrate. The optimal pH for halogenation is 3 and it has a turnover number for chlorine of 66,000. Between 3 and 6 isoenzymes are usually produced at the same time.⁶ Chloroperoxidase catalyses the classical peroxidation reactions which are characteristic of peroxidases. However, chloroperoxidase can also utilise chlorine, bromine and iodine ions as donors for enzymic halogenation reaction, a capability shown only by one other peroxidase, myeloperoxidase.⁷

Unlike other peroxidases, chloroperoxidase also resembles other non-peroxidatic enzymes. Many of the spatial and magnetic properties of chloroperoxidase closely parallel those of the cytochrome P-450 enzymes.⁸ Chloroperoxidase also catalyses the dissimilation of hydrogen peroxide and the oxidation of ethanol and formate, reactions which are generally characteristic of catalase.⁹

Thus in addition to halogenation (equation 1 above) CPO carries out :



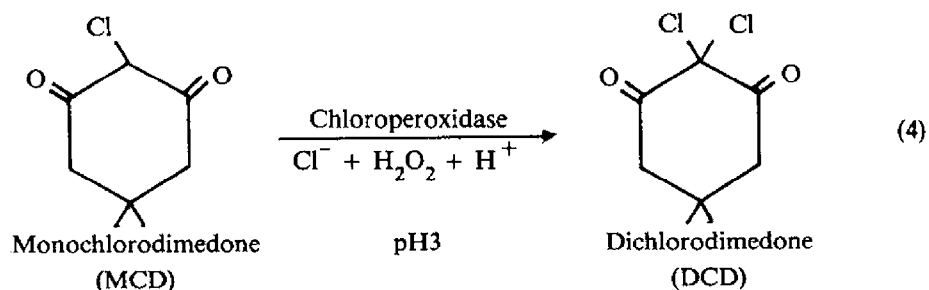
Chloroperoxidase widely reacts with alkenes, alkynes, cyclopropanes, phenols, anilenes and other aromatics, beta-diketones, beta-ketoacids, sulphur atoms, and even inorganic halogens other than halides.⁷

Recently chlorine oxidising peroxidases have been found which differ from the classical CPO in having pH optimas greater than 6.¹⁰ These enzymes were isolated

from several Dematiaceous Hyphomycetes, including members of the genera *Alternaria*, *Botrytis*, *Cladosporium*, *Dreschera*, *Embellisia*, *Helminthosporium*, *Stachybotrys* and *Ulocladium*.

2 Assay for Chloroperoxidase

Chloroperoxidase is assayed using monochlorodimedone in the presence of halide and hydrogen peroxide under suitable pH conditions (pH 3.0 for CPO), monochlorodimedone is converted to dichlorodimedone. The reaction is demonstrated below :



1 unit of CPO is defined as the activity that catalyzes the conversion of 1 μmol of MCD per minute, yielding an absorbance change of 12.2 per minute at 278 nm. The decrease in absorbance is measured spectrophotometrically.

3 Physiological and Genetical Optimization of Chloroperoxidase Production

Early studies of the extracellular production of chloroperoxidase from the sooty mould *Caldariomyces fumago* in the laboratories of the Institute for Biotechnological Studies showed a potent enzyme - producing system but with some problems. These included low growth rate, excretion of large amounts of a melanin-type pigment, pelleting of the mycelia, lowering productivity and interference in the ultrafiltration step of downstream processing.

To overcome some of these problems, a programme of mutagenesis (using FUV, NTG and X-rays) was undertaken and a range of 60 mutants selected initially as having reduced pigmentation by visual examination of the colonies growing on agar. Thirty-six of these mutants were then grown in batch 250 ml shake flasks. Figure 1 shows CPO activity and pigment content of the mutants. Five of the most promising mutants, CF16, CF59, CF60, CF506 and CF509 were chosen for further investigation in batch 2-litre fermentors on the basis of high enzyme titre, low melanin and the visual ability to sporulate.

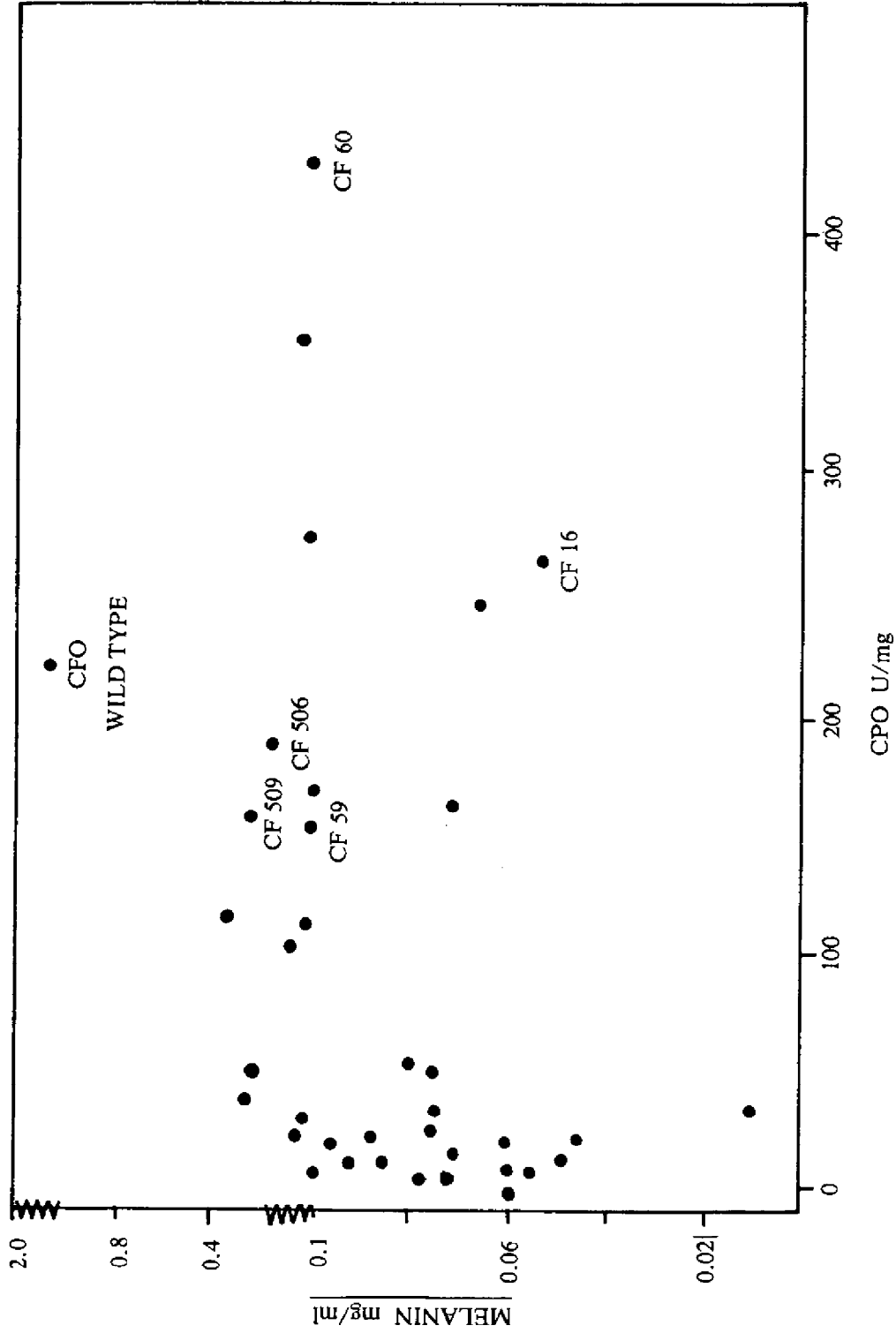


Fig. 1 Screening of *Caldariomyces fumago* CMI 89363 mutants

As can be seen in Figure 2 the wild type CFO has a growth rate of $\mu_{\max} 0.2h^{-1}$, produces $33 Uml^{-1}$ CPO and sporulates heavily. However, it synthesizes large amounts (3 mg/ml.) of a melanin-type pigment which was considered a problem for the proposed downstream processing by ultrafiltration. There was thus a need for a mutant with low melanin production and high CPO levels. Of the remaining mutants all but CF506 proved unsatisfactory on the basis of sporulation, growth rate or enzyme production (Figure 2). Isolate CF 506 was chosen as an 'improved' strain because of its comparable growth rate ($0.16h^{-1}$), increased spore production and 15 fold lower melanin levels when compared to the wild type.

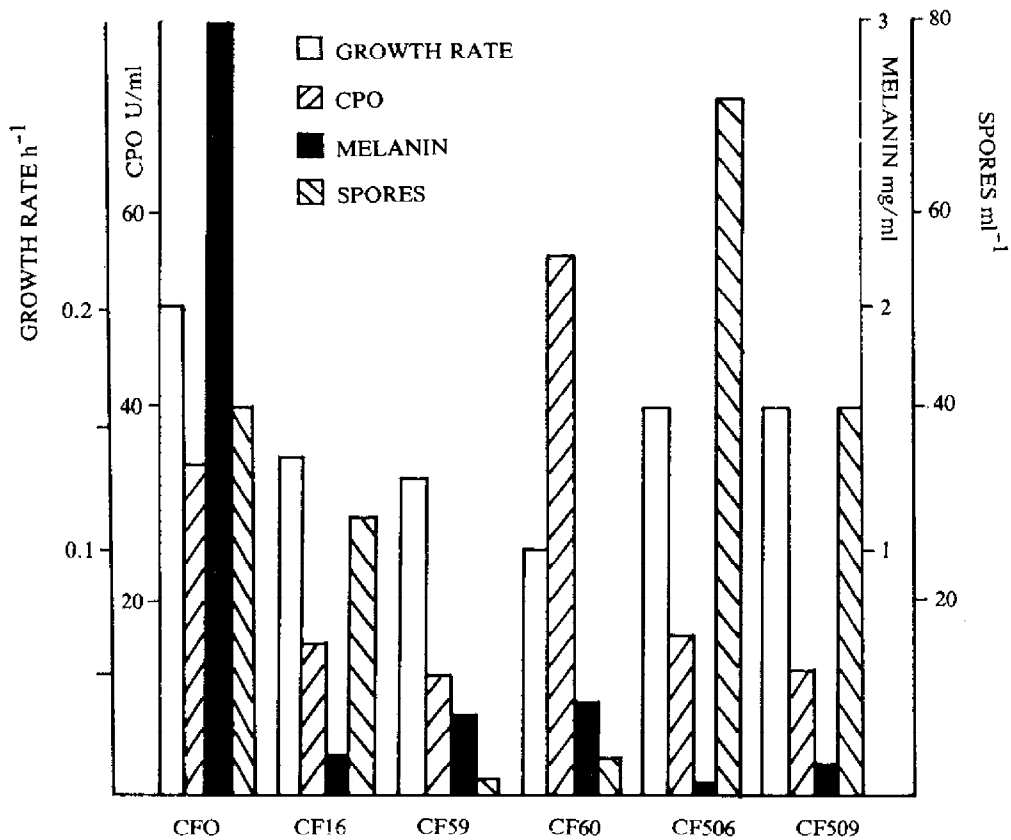


Fig. 2 Strain selection : Batch 2 litre fermentations

Early workers used essentially a modified Czapek-Dox media with fructose substituting for glucose (Media 1 in Table 2). This has been optimized to produce increased growth rate and CPO levels, while maintaining filamentous growth (Media 2 in Table 2). Very low growth rates and enzyme titres of 0.01h^{-1} and 0.05Uml^{-1} were obtained using the defined minimal media. The addition of trace elements to this defined media significantly increased both the growth rate and enzyme titres. Further improvement was gained by substituting the nitrogen source sodium nitrate (NaNO_3) with either ammonium chloride or urea. The addition of Yeast extract to this media further improved growth rate and enzyme titre (Figure 3).

TABLE 2 MEDIA DEVELOPMENT

MEDIA 1		MEDIA 2	
REAGENT	AMOUNT gl^{-1}	REAGENT	AMOUNT gl^{-1}
FRUCTOSE	40.0	YEATEX	10.0
KH_2PO_4	2.0	FRUCTOSE	10.0
NaNO_3	2.0	NaH_2PO_4	1.56
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1.0	NH_4Cl	2.5
KCl	2.0	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.25
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.02	CaCl_2	0.05
		$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	0.02
		$\text{ZnSO}_4 \cdot 4\text{H}_2\text{O}$	0.02
		$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.005
		$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.1
		Na_2SO_4	1.0
		EDTA	0.6

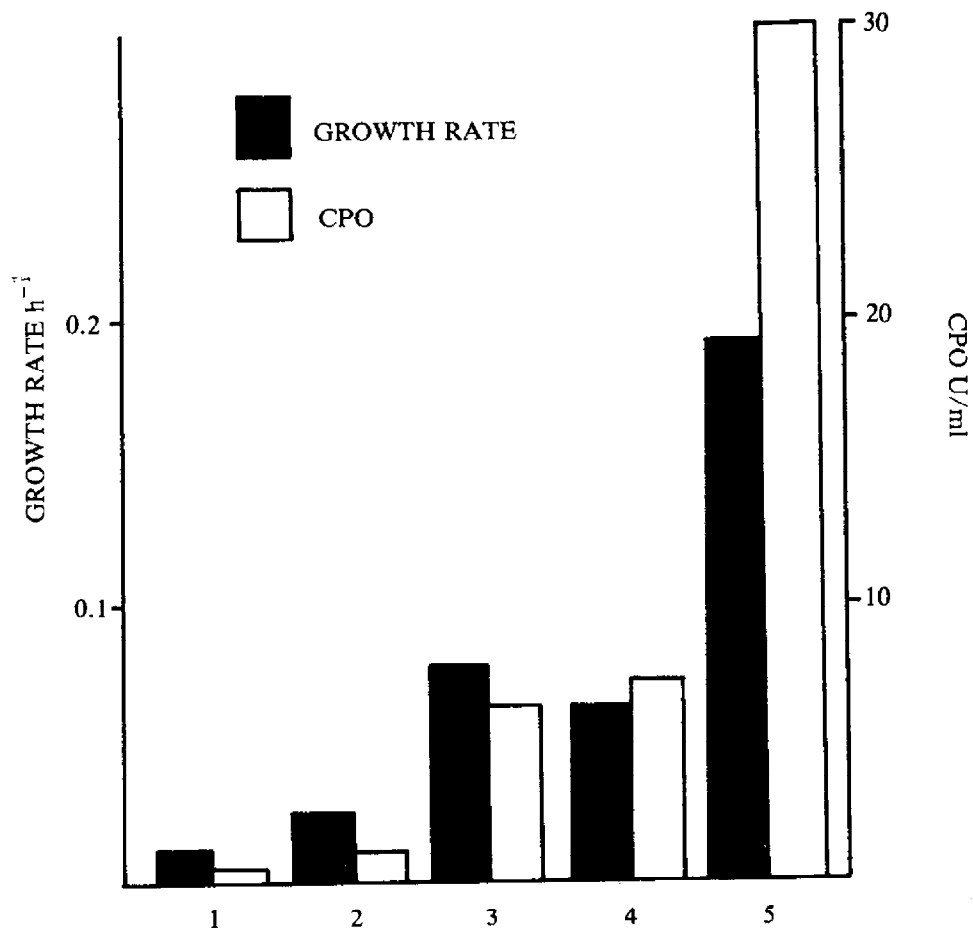


Fig. 3 Effect of media composition on growth rate and CPO production from *Caldariomyces fumago* in batch culture

1. Minimal media NaNO_3
2. Minimal media NaNO_3 trace elements.
3. Minimal media urea trace elements.
4. Minimal media NH_4Cl trace elements.
5. As 4 plus yeast extract.

It was also found that pelleting of the mycelia could be prevented by increasing the spore inoculum. Spore counts of $10^6 - 10^7$ spores ml^{-1} final concentration was optimal.

The optimal pH for reduced melanin is pH 5.0 and below (Figure 4). In terms of specific growth rate, enzyme titre and CPO stability, pH 5.5 can be considered optimal. Above pH 6 growth rate enzyme titre and stability are much reduced with significantly increased melanin levels. A temperature of 25°C was optimal for growth rate, enzyme titre, stability of CPO and reduced melanin (Figure 5). Either side of this value, reduced growth rate, CPO titre stability and an increase in melanin was observed.

4 Scale-up to 1000 Litre Production

Armed with data from the physiological optimization studies, the process was scaled-up to 1000 litres using the protocol detailed in Figure 6. Spores were harvested into minimal salts solution. An inoculum of 10^6 spores ml^{-1} was used as initial inoculum. 10% (v/v) growing culture was transferred at each stage. Tip speed of the impellers remained constant throughout the scale-up at 3 ms^{-1} . Standard growth media was used in all vessels, and was supplemented by an extra 1% fructose in the final production vessel. The mycelia were separated from the enzyme containing broth using a partial discharge disc stack centrifuge at a flow rate of 1500 l h^{-1} (Figure 7). The process time in the final production vessel was 48 h. Enzyme levels increased steadily to a maximum of 100 Uml^{-1} at 46 hours. This corresponded to depletion of fructose (Figure 6). Enzyme was produced throughout the growth period. The specific activity of chloroperoxidase was 600 umg^{-1} . During the fermentation the DOT (not shown) remained constant at 100% saturation for the first 23 hours and fell steadily to 76% at 46 hours followed by a rise due to the depletion of fructose. Pigment was produced throughout the growth period and reached a value of 0.25 mg ml^{-1} when the culture was harvested compared to 3 mg ml^{-1} for the wild type. The fact that the pigment was produced throughout the growth period suggests that it is not melanin being produced, since melanin is a classical secondary metabolite produced in idiophase.^{11, 12} The large lag period of 20 hours in the final production vessel may be due to either (a) osmotic shock since the production vessel contains twice the amount of fructose to the other seed vessels, or (b) the inoculation with a non-exponentially growing inoculum.

SCREENING FOR OTHER HALOPEROXIDASES AND NOVEL HALOGENATING SYSTEMS

Although there are numerous halometabolites, to date only one type of enzyme activity has been discovered. Such haloperoxidases have little substrate specificity and narrow pH spectra. The screen used in our studies has involved a bi-directional approach, screening for other haloperoxidases as well as for novel halogenating

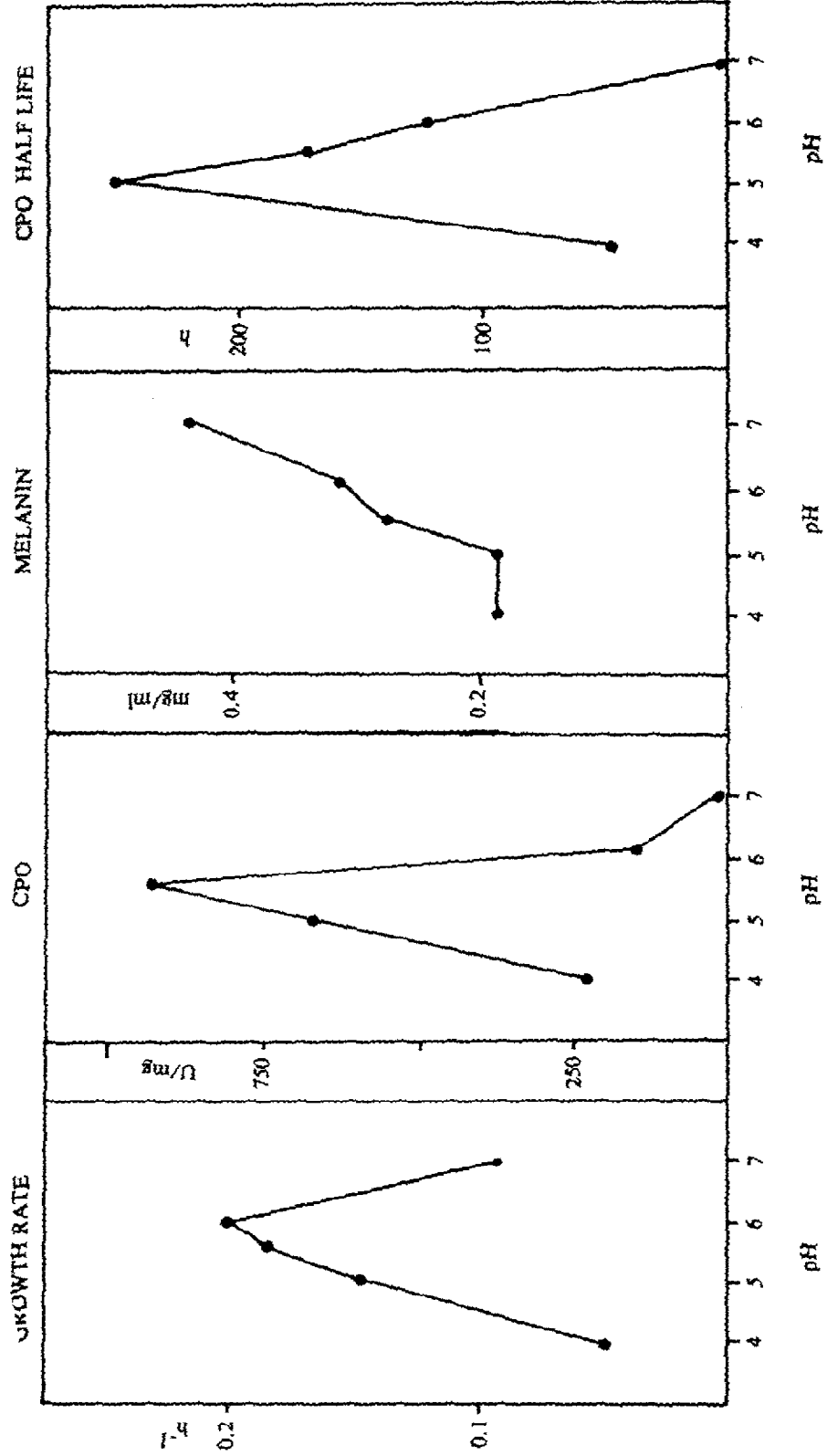


Fig. 4 Effect of pH on the batch fermentation of *Caldariomyces fumago*; temperature 25°C

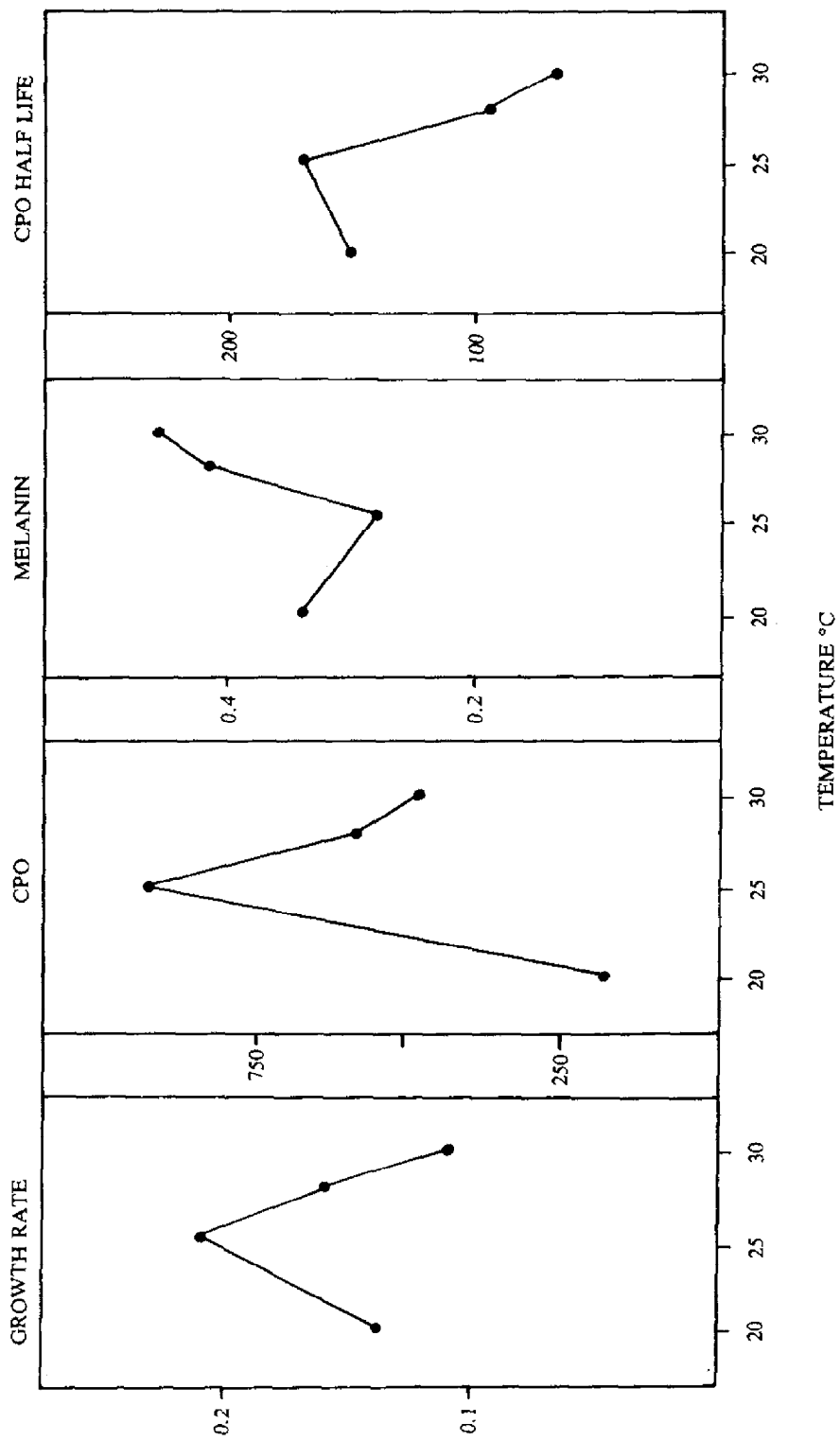


Fig.5 Effect of temperature on the batch fermentation of *Caldariomyces fumago*: pH 5.5.

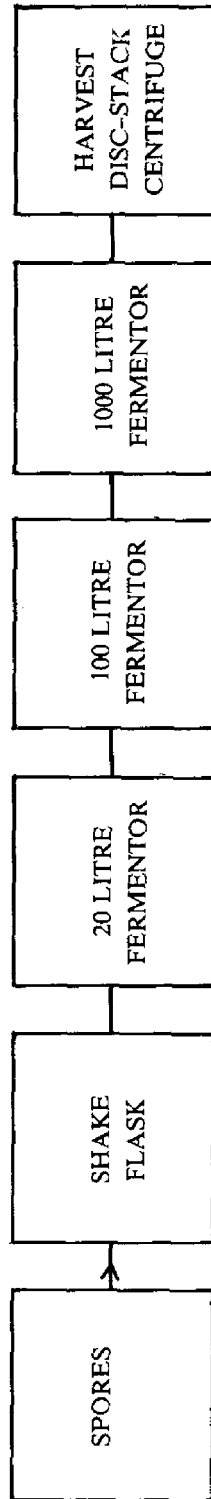


Fig.6 PROTOCOL: Scale-up and harvesting of chloroperoxidase from *Caldariomyces fumago* mutant CF506 fermentation.

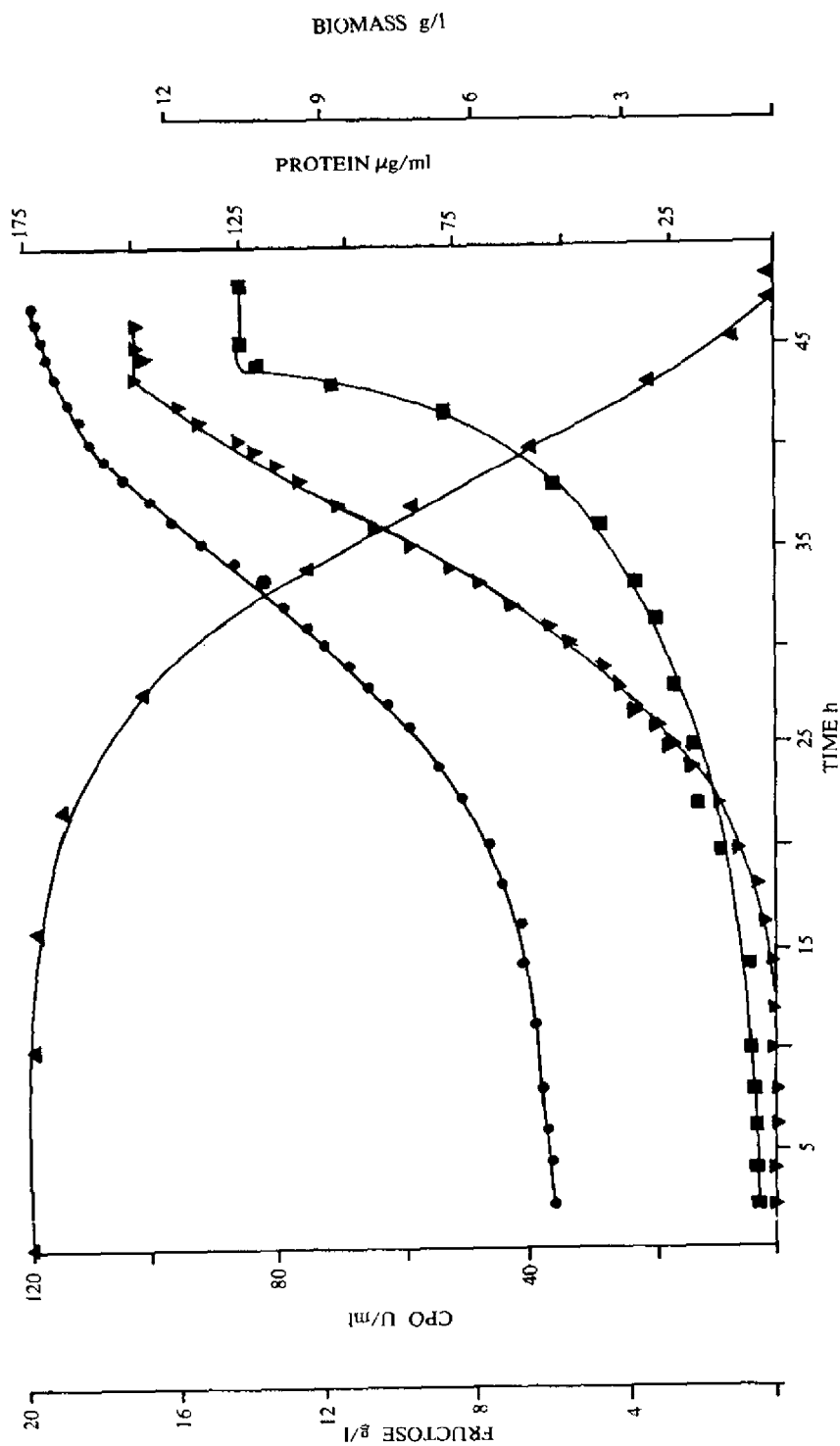


Fig.7 1000 I production fermentation of *Caldariomyces fumago* mutant CF506
 ▲Fructose g/l; ●Protein µg/ml; ▼CPO U/ml; ■Biomass g/l; pH 5.5;
 Temperature 25°C.

systems. Screening for other haloperoxidases has involved the development of a plate screen to enable CPO producers to be excluded. A number of dyes were investigated for the ability of CPO to change this colour. Of those investigated the triphenol group of dyes (including methyl green and zylene cyanol) were most promising and can be used routinely. Proof that the decolourization of the dye was due to halogenation was shown by employing autoradiography of reaction products following TLC.

Organisms not showing a positive on the plate screen were then grown in the presence of radioactive chlorine, Cl^{36} . Mini TLC's of culture broths and homogenized biomass have been run using nine different solvent systems ranging in polarity from polar to non-polar, and looking for incorporation of the radiohalide Cl^{36} into metabolites. On the basis of the number of potential metabolites isolated strains are being selected for further investigation of their ability to halogenate a range of model compounds. It is, however, worthwhile remembering that the final products from pathways that contain a halogenation step may not contain the halogen atom.

FUTURE DIRECTIONS

There are at least three reasons why peroxidases are important industrially. Firstly, halogenated intermediates are important in chemical synthesis.¹³ Secondly, halogenated compounds often confer high levels of biological activity (for example, hormones, steroids, pesticides and antibiotics)^{14, 15} and thirdly such enzymes can be widely employed in clinical diagnostics and treatment.¹⁶ Further research on these most fascinating halogenating enzymes (including, in time, application of protein engineering) will lead to new commercial opportunities. It might also throw light on the role of these enzymes in nature.

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