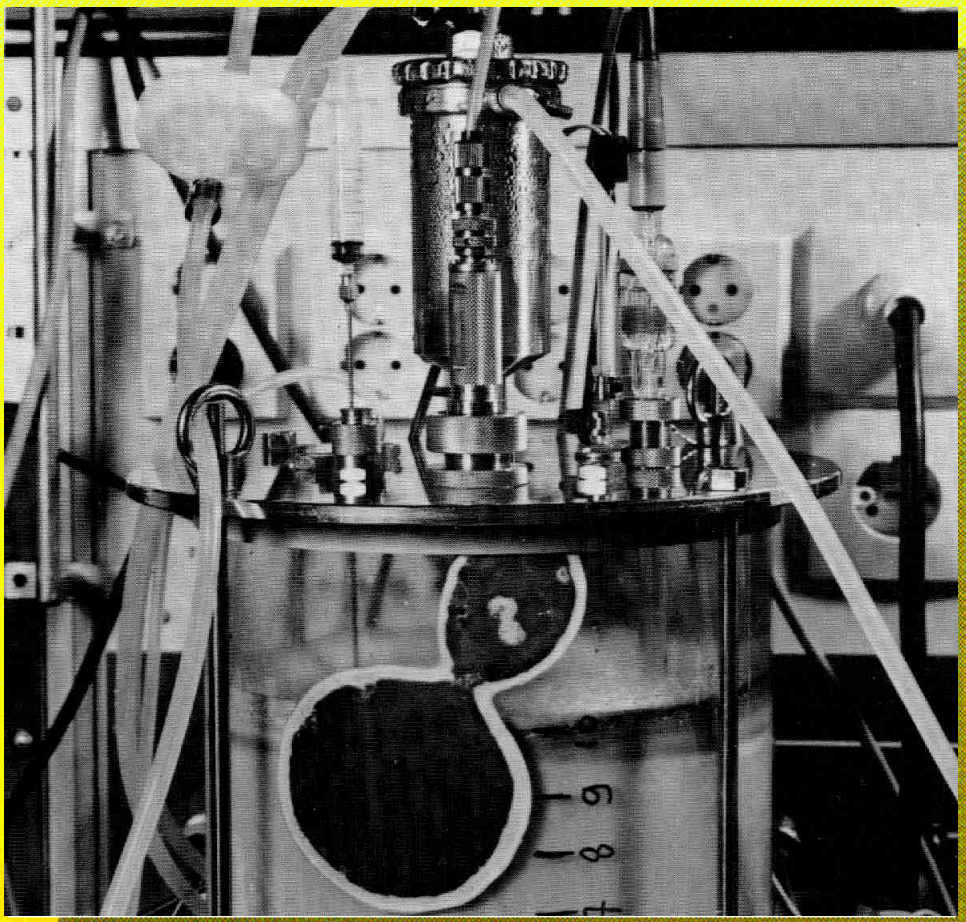
OPTIMIZATION OF METHANOL OXIDASE PRODUCTION BY *HANSENULA POLYMORPHA*

an applied study on physiology and fermentation

M.L.F. Giuseppin



Stellingen behorende bij het proefschrift van M.L.F. Giuseppin

1. - Ten onrechte wordt door Chander et al. aan citraat een stimulerend effect toegeschreven op de extracellulaire lipaseproduktie in schudkolfculturen van Rhizopus nigricans. In hetzelfde artikel wordt juist aangetoond dat niet het citraat, maar de calciumionen de lipaseproduktie stimuleren.
(Chander H., Batish V.K., Ghodekar D.R_ J. Dairy Sci. 64 (1981) 193-196).

2. - Elke stam van een micro-organisme die in een wetenschappelijk tijdschrift wordt beschreven, dient zonder restricties beschikbaar te zijn voor vakgenoten, hetgeen mogelijk gemaakt kan worden door het gebruik van centrale, open cultuurcollecties.

3. - Octrooien worden ten onrechte nauwelijks geciteerd in wetenschappelijke, biotechnologische publikaties.

4. - Het "detachment"-mechanisme beschrijft in slechts een beperkt aantal gevallen de snelheidsbepalende stap in de produktie van extracellulaire lipase in culturen van gram-negatieve bacteriën.
(Winkler u.K., Stuckmann H., J. Bacteriol. 138 (1979) 663-670.; Schulte G., Bohne L., Winkler K., Can. J. Microbiol. 28 (1982) 636-642).

5. - De omzetting van isopropanol naar aceton door op methanol gekweekte Hansenula polymorpha wordt ten onrechte geheel toegeschreven aan alcohol-dehydrogenase-activiteit.
(Hou C.T., Patel R., Laskin A.I_ Barnabe N., Marczak I., Appl. Environ. Microbiol. 38 (1979) 135-142.; Huang T-L, Fang B-S, Fang H-Y, J. Gen. Appl. Microbiol. 31 (1985) 125-134).

6. - *Ondanks de complexe regulatie van het methanolmetabolisme in methylotrofe gisten kan een eenvoudig inductie- en repressiemechanisme de groei van en de methanoloxidase-produktie door Hansenula polymorpha onder reëele procescondities voldoende beschrijven ten behoeve van initiële procesoptimalisatie.*
(Dit proefschrift)

7. - De vermelde vetzuursamenstelling en het substraatgebruik duiden erop dat 7.-Tahoun et al. in hun experimenten géén Candida lipolytica hebben gebruikt, zoals zij veronderstellen.
(Tahoun M., Shata 0., Mashaley R., Abou-Donia S., Appl. Microbiol. Biotechnol. 24 (1986) 235-239).

8. - Het is onjuist en misleidend de expressie van genprodukten uit te drukken als de concentratie van het produkt in de cel of bet medium. De produktiesnelheid per hoeveelheid biomassa is een betere maat voor de expressie.
(b.v. Tschopp J.F., Sverlow G., Kosson R., Craig W., Grinna L. Biotechnology 5 (1987) 1305-1308).

9. - In studies op het gebied van de produktíe van extracellulaire enzymen (bijv. Lipases en proteases) wordt te weinig aandacht besteed aan de effecten die optreden bij de hoge enzym- en biomassaconcentraties die bij produktieprocessen worden nagestreefd.

10. - De thans veel gebruikte, ongestructureerde modellen voor het beschrijven van de groeisnelheid en de aanloopfase ('lag phase') van micro-organismen als functie van de temperatuur, zijn niet geschikt voor een verantwoorde risico-analyse van infecties in voedingsmiddelen bij extreem lage temperaturen.
(Schoolfield R.M., Sharpe P.J.H., Magnuson C.E.J., Theor. Biol. (1981) 88 719-731; Ratkowsky D.A., Lowry R.K., MeMeekin T.A., Stokes A.N., J. Bacteriol. 154 (1983) 1222-1226; Broughall J.M., Brown C., Food Microbiol. 1 (1984) 13-22).

11. - Voor een goedkoop en stabiel enzym vindt men altíjd wel toepassing.

12. - Ondanks de reclameboodschappen die beweren dat het wasgoed met een goed "biologisch" wasmiddel schoon, wit en klaar uit het sop komt, is het allesbehalve klaar wat zich fysich-chemisch gezien afspeelt in de was.

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an applied study on physiology and fermentation

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Technische Universiteit Delft, op gezag van de Rector Magnificus, Prof. dr. J.M. Dirken, in het openbaar te verdedigen ten overstaan van een commissie aangewezen door het College van Decanen op donderdag 2 juni 1988 te 14.00 uur

door

MARCO LUIGI FEDERICO GIUSEPPIN

geboren te 's-Gravenhage

Dit proefschrift is goedgekeurd door de promotor
PROF. DR. J.G. KUENEN

GRAFISCHE VERZORGING
John Deij,
Petra van Everdingen,
Cor Gardien,
Mari van der Giessen.

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1. INTRODUCTION

1.1 Context and aim

General.

Recent developments in biological and engineering sciences have a great impact on the interdisciplinary field of biotechnology. In this framework biotechnology deals with the integral application of biochemistry, (micro-) biology and (bio-) process technology in biological systems in order to design and improve industrial processes and environmental control. In this respect, there have been important developments in molecular biology and genetics, which enable transfer and modification of genes, that code for valuable products, such as enzymes. However, not only the new techniques available in molecular biology have led to the present state of the art in biotechnology. Also, for example, new methods in biochemistry, using the latest physical chemical techniques and powerful computers, have increased the insight on the mechanism of enzyme action. This improved knowledge is being used to modify the properties of enzymes. In addition, the purification methods for small-scale and even large-scale have been substantially improved.

In most biotechnological processes microorganisms are used for production and catalysis. The growing knowledge on the physiology of microorganisms also contributes significantly to the present development of biotechnology. This knowledge on microbial physiology provides essential information on the metabolic and biosynthetic processes in the cell, and their dependence on environmental factors.

The combination of the above-mentioned scientific fields has enabled the manipulation of microorganisms on various levels. This manipulation can easily be compared to engineering; the sub-disciplines in biotechnology are therefore described as genetic-engineering, enzyme-engineering, metabolic engineering etc.

Last but not least, the important contribution of bioprocess engineering must be mentioned. Biochemical engineers are needed to tailor biological or biochemical reactions to the requirements of large scale processes. They use chemical engineering principles and applications of these principles for process scale-up and design of process control. New technologies have been developed to study the complex processes involving biological materials, including the modelling of the process at the microbial level (microkinetics) and at the bioreactor level (macrokinetics). In this way, the modelling of the processes has led to sophisticated methods for scaling up and bioprocess control.

The above-mentioned developments in biotechnology enable industries the production and application of special biological products (e. g. enzymes) on industrial scale at relatively low costs. Many enzymes have come onto the market, and new enzymes are being developed for application in a great variety of processes and products.

Application of oxidases.

In our laboratory enzymes e.g. proteases and lipases, have been studied for many years with respect to their use in detergent formulations. Presently, the possible use of oxidases in detergents is of interest. Oxidases catalyse the oxidation of a substrate under the formation of hydrogen peroxide. The generation of hydrogen peroxide may improve the "bleaching" performance of the detergent.

Oxidases may have many other potential applications in various fields including both small and large scale operations (Woodward, 1986). Many applications of oxidases have already been studied and described in patent literature. A few examples will be given to illustrate the wide range of applications.

Oxidases can be used to generate hydrogen peroxide at low temperatures as a bleach precursor in detergents (Unilever, 1983, 1986, 1987a, b; Henkel, 1977).

For these applications, the oxidase preparation must be free of any catalase activity, to avoid premature decomposition of the hydrogen peroxide formed. The potential market volume for this type of application may be as large as the present protease market volume, provided that the production costs for catalase-free oxidase are low and that the enzyme is compatible with the other detergent components.

- Oxidases can be used for analytical purposes. The substrates of oxidases can be detected even at very low levels by using reactions coupled to the oxidase reaction. The coupled reaction is used to generate a coloured compound (Verduyn et al., 1984; Herzberg and Rogerson, 1985). Such a coupled reaction can consist of an oxidase and a peroxidase e.g. horse radish peroxidase. Peroxidase decomposes hydrogen peroxide, produced by the oxidase, and is able to oxidize a dye, which results in a change of color. In this way ethanol (Phillips Petroleum Co., 1980), lactate (Eastman Kodak Co., 1985) and glucose can be determined in routine analysis e.g. for clinical purposes. A well-known example is the glucose assay dip stick (based on glucose oxidase), which is used routinely by diabetics to test the glucose level in the blood.
- Oxidases may be used to scavenge traces of oxygen in certain products, such as foods, to improve their keeping ability and taste (Behringwerke, 1974). This application area is growing fast, having a relatively high potential market value.
- Even environmental applications have been patented e.g. the use of oxidases in enzyme mixtures to cleanse particular waste waters (Phillips Petroleum Co., 1984b). Oxidases offer alternative ways to decompose compounds, that are converted slowly by microbial systems used in waste water treatments, although the current costs of oxidases will limit this field of application.
- There is a growing interest in using oxidases for the synthesis of various organic compounds, mainly on the scale of fine-chemicals. Synthesis of aldehydes from alcohols by methanol oxidase or other oxidases have already been reported e.g. formaldehyde and acetaldehyde

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(Kato et al., 1983; Tani et al., 1985a,b; Sakai and Tani, 1987). Here too, the cost of the enzyme limits the scale of production. Furthermore, the stability of the enzyme must be improved to enable high product concentrations in these processes.

- The reaction of oxidases e.g. methanol oxidase, can be coupled to other enzyme reactions in order to produce compounds such as ATP (Yonehara and Tani, 1987), or formic acid (Mizuno and Imada, 198).
- Oxidases in intact microorganisms may also be used for conversion processes e.g. the commercial production of gluconic acid by Aspergillus niger, which proceeds via oxidation of glucose by glucose oxidase.

My study mainly focused on the potential use of methanol oxidase (MOX) (E.C. 1.1.3.13) as a hydrogen peroxide generating system in detergent formulations. The name methanol oxidase rather than the official name alcohol oxidase (AO) (Webb, 1984) will be used thoughout this thesis in view of the physiological function. MOX catalyses the oxidation of alcohols, forming aldehyde and hydrogen peroxide:

$$R - CH_2 - OH + O_2 \rightarrow R - \underset{H}{C=O} + H_2O_2$$

The group R is preferentially $H-(CH_2)_n$ with n = 0,1,2,3 or an other small group.

Source of MOX.

Methylotrophic yeasts are a useful source of methanol oxidase, also from a commercial point of view (Lee and Komagata, 1983). These yeasts can use methanol as the sole carbon source, which is not very common among these organisms (Veenhuis et al., 1983b). The occurence of MOX has been described in some filamentous fungi (Janssen and Ruelius, 1968; Bringer et al., 1979; Bringer, 1980) and in yeast species of the genera Hansenula (van Dijken et al., 1976), Pichia (Patel et al., 1981) and Candida (Torulopsis and Kloeckera) (Tani et al., 1972; Yamada et al., 1979; Unichika Co., 1986;Egli, 1980). In this thesis the filamentous fungi will not be discussed as they are a less suitable source of MOX. They offer low levels of MOX, have a low growth rate and are difficult to cultivate compared with yeasts. Many aspects of the physiology of the taxonomically-closely-related methylotrophic yeasts have been studied in the past decade and several reviews on this subject have appeared (Veenhuis et al., 1976; Harder et al., 1987). More information on the physiology will be given in Section 1.2.

Initially, most studies on the physiology of methylotrophic yeasts were carried out in the framework of single cell protein (SCP) production (Cooney et al.,1975; Cooney and Levine, 1975; Cooney and Swartz, 1982; Levine and Cooney, 1973). However, due to the present high prices of oil and methanol, there is only little interest for this application. Companies formerly involved in this field now use their SCP production technology to make proteins with a high added value in an inexpensive way. This has been applied to both yeast systems (Phillips Petroleum Co., 1983, 1984a; Cregg et al., 1987) and bacterial systems (e.g. Hoechst, 1974, 1984).

Choice of microorganism.

There are various reasons why the yeast Hansenula polymorpha was chosen as a source of MOX in this study. In the first place, MOX produced by H. polymorpha has favourable properties for applications in a detergent system. Its temperature optimum, thermostability and stability in liquid detergents is good compared to that of other methanol oxidases (Unilever, 1983). In the second place, the yeast itself has been studied in great detail for many years by several groups, thus providing a good scientific basis for the development of a production process with this organism.

Apart from these considerations, H. polymorpha, like related yeasts, seems a potential host for foreign (heterologous) genes. This option is offered by the possibility to use the strong genetic regulatory elements and promotors that are involved in the formation of enzymes for the methanol metabolism (Unilever, 1986). Indeed, a considerable yield of

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recombinant DNA product was obtained in Pichia pastoris, a yeast closely related to H. polymorpha. Using P. pastoris as a host organism, Cregg et al. (1987) found expression levels of heterologous genes of 2 up to 10% of the soluble intracellular protein. Also high heterologous expression of extracellular invertase has been reported for an expression system using P. pastoris as a host organism (Tschopp et al., 1987). On the basis of the similarities between Pichia and Hansenula with respect to their physiology of methanol metabolism and the occurence of strong promotors, high expression yields of heterologous genes may also be expected for H. polymorpha.

Aim.

The aim of this thesis was to study the relevant physiological and fermentation characteristics of H. polymorpha in order to optimize the MOX-production process. The optimization strategy for the microbiological part of the project was formulated as the study of the physiological factors that determine the specific productivity of the fermenter, the recovery of MOX and the stability of the culture. In addition, a mathematical model was developed. This enables the description of the essential fermentation process phenomena involved in the production of MOX. This simple model may be used for analysis and initial optimization of the process by simulation techniques. It may also serve as a basis for further scale-up of the process to large-scale fermentation, including adequate process control.

In the following sections of this chapter (1.2 - 1.6) various aspects of MOX production by H. polymorpha will be discussed to show the potential bottlenecks in the optimization of the process. These bottlenecks, which may limit the productivity, the specific activity and/or the applicability of MOX, will be discussed in terms of: the physiology of growth on methanol, the mechanism of MOX formation (induction and biosynthesis) and the biochemical characteristics of MOX. Finally, process stability, cell wall strengtH. and engineering limitations will be discussed.

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1.2 Physiology of Hansenula polymorpha related to the metabolism of methanol

H. polymorpha, like all other yeasts capable of using methanol as the sole carbon and energy source, is a facultative methylotroph; it can grow equally well or better on a large variety of substrates other than methanol. The physiology of methylotrophic yeasts has been studied extensively and the enzymes involved in methanol metabolism have all been identified and characterized (Egli et al., 1983). It has been shown that all the methylotrophic yeasts share an identical metabolic pathway for methanol.

The metabolism of methanol and other C-1 compounds by yeasts turned out to be considerably different from that of bacteria. Bacteria were found to use three types of metabolic pathways for C-1 compounds. The ribulose bisphosphate cycle, the ribulose monophosphate cycle and the serine pathway for bacteria were established (Kato et al., 1983). In bacteria the methanol is initially oxidised by methanol dehydrogenase, which contains either NAD or PQQ as a cofactor (Duine et al., 1987).

Outline of routes for methanol metabolism in yeasts,

In contrast to bacteria, the oxidation of methanol to formaldehyde by yeast does not yield useful energy since this reaction, as mentioned above, is catalysed by an oxidase (MOX). This first key enzyme for methanol metabolism is situated in specialized organelles called peroxisomes (Fukui and Tanaka, 1979). A schematic drawing of methanol metabolism in the peroxisomes is given in Fig. 1. In the peroxisomes MOX is arranged in a highly regular structure, which appears as a crystalline pattern in electron microscopic photographs (Veenhuis et al., 1976).

Not only MOX, but also catalase (Fig. 1) is present in these regular structures. High levels of catalase activity are needed to detoxify the hydrogenperoxide formed in the MOX-mediated reaction. The compartmen-

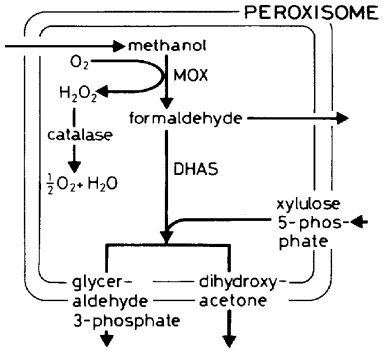

Fig. 1 : Metabolism of methanol in the peroxisome.
MOX, methanol oxidase; DHAS, dihydroxyacetone synthase.

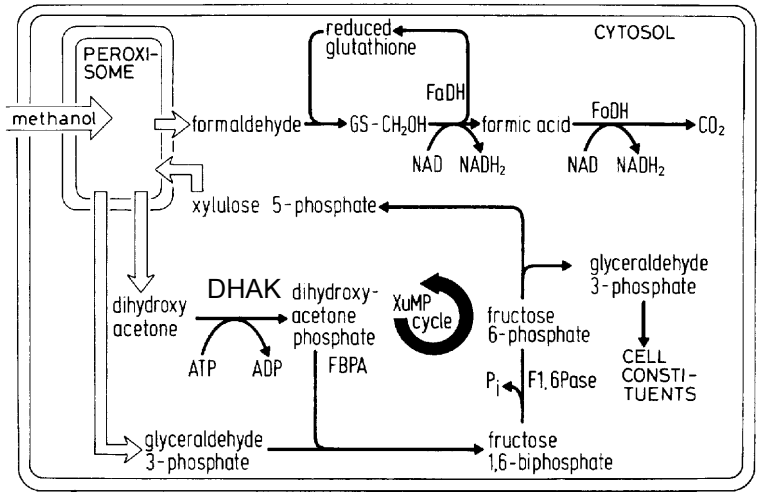

Fig 2 : Schematic representation of methanol metabolism in yeasts.
After Douma et al., 1985.
ATP, adenosine triphosphate; DHAK, dihydroxyacetone kinase;
FaDH. formaldehyde dehydrogenase; FoDH. formate dehydrogenase;
F1,6 Pase, fructose 1,6-biphosphatase; FBPA, fructose 1,6-biphosphate aldolase; NAD, nicotineamide adenine dinucleotide; XuMPC, xylulose monophosphate cycle.

tation of MOX and catalase in the peroxisomes protects the cytosol against the negative effects of the reactive hydrogen peroxide.

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Therefore it was thought that catalase was essential for growth on methanol containing media (Eggeling and Sahm, 1980). However, it has been recently shown that H. polymorpha contains an alternative system to decompose hydrogen peroxide. This system is based on cytochrome C peroxidase, which is induced in response to formation of low levels of hydrogen peroxide in the cytosol (Verduyn et al., 1987). This alternative hydrogen peroxide-decomposing system is situated in the cristae of the mitochondria. This system enables a catalase-negative mutant of H. polymorpha to utilize methanol in methanol/glucose mixtures (Giuseppin et al., 1988b). Chapter 4 describes this phenomenon in more detail.

The MOX-mediated reaction yields formaldehyde, the compound on the branch-point in the C-1 metabolism. Formaldehyde is either dissimilated or assimilated. After its diffusion into the cytosol, dissimilation occurs via a NAD-and GSH-dependent formaldehyde dehydrogenase and a NAD-dependent formate dehydrogenase (Fig. 2). In contrast to C-1 metabolism in bacteria, assimilation of formaldehyde occurs via the xylulose monophosphate (XuMP) cycle (van Dijken et al., 1978; Waites and Quayle, 1980). The XuMP-cycle is unique for yeasts and does not occur in bacteria (Veenhuis et al., 1983b). Dihydroxyacetone synthase (DHAS), a special transketolase, which is also situated in the peroxisome (Douma et al,, 1985), is the key enzyme for the assimilation of formaldehyde by this cycle (Fig. 1). The localization of DHAS in the peroxisome is one of the reasons why the assimilation of formaldehyde can only be accomplished with formaldehyde generated in the peroxisome; formaldehyde supplied extracellularly is completely converted via the dissimilatory route in the cytoplasm (Egli, 1980). Apparently the scavenging of extracellular formaldehyde by the formaldehyde dehydro- genase system (Fig. 2) is so efficient that this compound does not reach the peroxisomes.

In the XuMPcycle, glyceraldehyde 3-phosphate and dihydroxyacetone, formed by the action of DHAS, are transported to the cytosol (Fig. 1). There they are converted into xylulose 5-phosphate and glyceraldehyde 3- phosphate. The latter compound is used for biosynthesis of the cell components. The xylulose 5-phosphate is recycled into the peroxisome.

The net reaction of this cycle can be described as:
3 formaldehyde + 3 ATP → glyceraldehyde 3-phosphate + 3 ADP + 2Pi

Regulation of methanol metabolism.

Studies on the regulation of the MOX mediated reaction and the XuMP-cycle have shown that several mechanisms may contribute to the control the carbon fluxes during methanol metabolism (Harder et al., 1987).These mechanisms can be divided into two types of regulation, depending on the time scale of the action. A short and long term regulation can be distinguished. The short term regulation occurs by direct control of metabolic fluxes. The long term regulation occurs either by de novo enzyme synthesis or by catabolite inactivation. The long term regulation will be discussed in Section 1.3.

The short term regulation of metabolic fluxes across the peroxisomal membrane may consist of various feedback mechanisms. These are assumed to involve allosteric effects of various metabolites on key enzymes (Harder et al., 1987). A high energy level in the cell, characterized by a high level of NADH or ATP, causes a decrease of the dissimilation via formaldehyde dehydrogenase. This is due to the fact that both NADH and ATP are inhibitors of formaldehyde dehydrogenase. On the other hand, high levels of ADP and AMP inhibit dihydroxyacetone kinase activity (Fig. 2). This inhibition implies that under conditions with low energy levels, i.e. low ATP potential, more formaldehyde becomes available for the dissimilation via formaldehyde dehydrogenase. This increase of the flux through the dissimilation route gives rise to more NADH and subsequently more ATP. It can be seen that in this way the reactions form a closed feedback control loop.

Another type of short term effect leads to the direct inhibition of the formaldehyde flux. When high concentrations of methanol are present in the medium, MOX is inactivated by the high level of formaldehyde formed by the MOX mediated reaction (Veenhuis et al., 1976; Fuji et al.,

1984). Depending on the extent of formaldehyde accumulation, other enzymes may be damaged as well, resulting in the death of the cell. It is evident that this type of effect can not be regarded as a physiological adaptation. An alternative way to control the formaldehyde flux may be obtained via the NADH-dependent formaldehyde reductase found in e.g. Pichia pastoris. It has been suggested that under conditions with high levels of formaldehyde in the cell (e.g. after a pulse of methanol to a culture) the formaldehyde reductase generates methanol (Hou et al., 1982). This may be regarded as a detoxification reaction since methanol is less toxic than formaldehyde. This type of regulation has not been confirmed experimentally sofar. It seems unlikely that this enzyme plays an important role in methanol metabolism, since the formaldehyde reductase of Pichia pastoris, for example, has a low affinity for formaldehyde (16.9 mM).

Growth on mixtures of methanol and other carbon sources.

The allosteric feedback mechanisms described above may not only control metabolic fluxes during growth on methanol, but also in cultures utilizing mixtures of methanol and glucose or sorbitol (Egli, 1980; Egli et al., 1987; Eggeling and Sahm, 1981). It was shown that within a wide range of dilution rates the fraction of methanol that is dissimilated remains constant at about 61%. At high dilution rates and at high methanol/glucose ratios the consumption of methanol turned out to be limited to a maximum value of 0.42 to 0.44 g methanol · (g dry weight biomass)$^{-1}$·h^{-1}. This maximum value is close to the maximum value for methanol consumption in a continuous culture grown on methanol as the sole carbon and energy source. This maximum methanol consumption rate can probably be ascribed to the limited assimilation capacity of DHAS (Egli et al., 1983). In general, it can be seen that the growth of H. polymorpha and other methylotrophic yeasts is restricted to low rates not higher than 0.22 h^{-1} (Levine and Cooney, 1973; Urakami et al., 1983; Urakami and Takano, 1984), which possibly reflects the limited capacity of DHAS. By using mixtures of methanol and glucose as a feed for continuous cultures of H. polymorpha it is possible to increase the

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maximal growth rate up to the rate for growth on glucose, with the complete consumption of methanol. It should be noted that also under those conditions the maximal methanol consumption rate is restricted to the above mentioned maximum value of 0.42 to 0.44 g methanol·(g dry weight biomass)$^{-1}$·h^{-1}. Higher methanol feed rates in continuous cultures result in the accumulation of methanol in the medium (Egli et al., 1987).

The simultaneous metabolism of methanol and glucose requires a welltuned control of the carbon flows (Egli et al., 1983) and the energy flows. In that respect it should be noted that during growth on methanol the energy is generated by the oxidation of the cytosolic NADH (Fig. 2) and that the tricarboxylic acid (TCA) cycle is mainly used for biosynthetic purposes (Egli and Lindley, 1984). An increased fraction of glucose in the feed of the continuous culture results in a gradual shift towards energy generation by oxidation of NADH. which is generated by the TCA cycle. This indicates the regulation of the energy flow (Egli and Lindley, 1984).

The physiological need for high levels of MOX.

In view of the desirability to increase the amount of MOX in the cell for production purposes it is essential to know the physiological role of large quantities of MOX in the cell, which may account for up to 70% of the cell volume or up to 37% of the cellular soluble protein. It has been observed that the MOX activity in cell-free extracts can account for more than twenty times the maximal steady state metabolic turnover of methanol (van Dijken et al., 1976; Giuseppin et al., 1988c). This has led to many speculations on yet another role of MOX besides the oxidation of formaldehyde (Hopkins and Mueller, 1987). In the following physiological conditions. The physiological conditions in continuous cultures are characterized by low methanol concentrations of about 31-94 μM (Egli et al., 1983, 1986). Assuming that the culture is grown on methanol at a dilution rate of 0.1 h^{-1} with a biomass yield on methanol of 0.4 g·g^{-1}, the methanol consumption rate will be 7.8 mmol methanol·g biomass^{-1}·h^{-1}. The maximum MOX activity in the cell is assumed to be equal to the MOX activity measured in cell-free extracts. This maximum MOX activity is about 12 Units per mg protein (van Dijken et al., 1976) in an air saturated buffer at 37°C (oxygen concentration is 0.216 mM). The actual MOX activity in the cell

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(V) can be described by a two substrate dependent reaction kinetics (e.g. Lehninger, 1975):

$$V = \frac{V_{max}}{1 + \frac{Km,O_2}{[O_2]} + \frac{Km,methanol}{[methanol]} \cdot \left[1 + \frac{K}{[O_2]}\right]}$$

The constants Km,0_2 and Km,methanol are the affinity constants for respectively oxygen and methanol under conditions of saturation of the other substrate. An estimate of these constants are respectively 0.4 and 2.8 mM (van Dijken et al., 1976). The constant K in this formula is the dissociation constant for oxygen with the enzyme. K is 0.13 mM. Its value can be calculated from data reported by van Dijken et al. (1976). It should be noted that this formula gives too high and optimistic estimate of V_{max} because various negative effects on the MOX activity such as diffusion limitations across the membrane and in the crystalline structure of MOX are neglected. With this formula the actual methanol conversion rate by MOX can be calculated as a function of the dissolved oxygen concentration and the residual methanol concentration. From this equation it can be seen that at low methanol concentrations, the methanol-term in the denominator has a predominant effect on the enzyme activity. At low methanol concentrations, a decrease of the oxygen concentration in the range of 50 to 10% air-saturation has no significant effect on the reaction rate. With the above equation and the assay conditions for MOX (37°C, 0.216 mM 0_2, 100 mM methanol) a V_{max} of 931 mmol methanol·g biomass^{-1}·h^{-1} can be calculated (biomass contains about 45% protein). At an oxygen concentration of 50% air saturation at 37°C (108 mM) the methanol conversion rate by MOX is dramatically reduced to 2, 6.4 and 12 mmol methanol-g biomass^{-1}·h^{-1} at residual methanol concentrations of 31, 62 and 94 μM, respectively. The methanolterm in the denominator of the two-substrate dependent reaction kinetic equation has a predominant effect at the low methanol con-centrations. Therefore a decrease of the oxygen concentration down to

25% air saturation has no significant effect on the methanol conversion rates. These calculations show that under physiological conditions the actual methanol conversion rate by MOX is only in a slight excess compared with the methanol feed rate of 7.8 mmol methanol·g biomass^{-1}·h^{-1}. Therefore it is essential for the yeast to make large quantities of MOX to assure a sufficient flux of formaldehyde. Using the above estimates, the required MOX concentration will be in the range of 25 to more than 48% of the cellular protein, which are in fact the ranges of MOX levels found in H. polymorpha.

1.3 Induction and repression of methanol oxidase synthesis

A well-tuned regulation of the synthesis of enzymes involved in methanol metabolism, is essential for the facultative methylotrophic yeasts to adapt effectively to their environment. In nature the adequate adaptation to methanol metabolism is essential in the competition with other organisms in environments with methanol. On the other hand, the enzymes involved in the methanol metabolism must be quickly broken down when methanol is no longer available and alternative substrates are provided. These examples are, of course, two extremes in the regulation of methanol utilization. In general the synthesis of the enzymes involved in methanol metabolism can be described in terms of induction and derepression. The negative control of enzyme levels takes place by repression and catabolite inactivation, which represent the long-term regulation of methanol metabolism.

When, in batch cultures grown on glucose or ethanol, no or a very low level of MOX activity is detected, the synthesis is called repressed. However, when the glucose is exhausted, low levels MOX activity are found as a result of a relief of this repression. This phenomenon is called derepression. The synthesis of some other enzymes, e.g. formaldehyde dehydrogenase and formate dehydrogenase, have also been found to be (de-)repressed in this way (Sahm, 1973; Eggeling et al., 1977; Eggeling and Sahm, 1980). Similar results have been reported for continuous cultures of H. polymorpha grown on glucose. At low dilution rates some MOX activity is detected, whereas at high dilution rates, characterized by relatively

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high glucose concentrations, the amount of MOX drops to levels normally found for repressed conditions (Egli et al., 1980).

Induction and derepression of MOX.

High levels of MOX in H. polymorpha are found when the cells are grown on methanol in both batch and continuous cultures. As the resulting activity is more than ten times as high as that found under derepressed conditions, this phenomenon can be interpreted as super-derepression or induction (Eggeling and Sahm, 1980). Here, this effect will be called induction, although the exact mechanism is not clear yet. This induction phenomenon is also observed when the cells are incubated batchwise with formaldehyde or formate, the intermediates of the dissimilation of methanol (Eggeling and Sahm, 1978). In continuous cultures, too, mixtures of either formaldehyde or formate with glucose as carbon source can lead to an effective induction of MOX in the cell (Giuseppin et al., 1988a).

Although the presence of methanol results in a high level of induction of MOX and other enzymes for methanol metabolism it is still not exactly known what compound acts as the actual inducer. In this context an inducer is defined as a compound that interacts with a specific protein (e.g. repressor) or protein complex controlling the efficiency of transcription of the structural gene. From batch culture studies on MOX-negative mutants of H. polymorpha it was concluded that methanol rather than formaldehyde and formic acid is the inducer (Eggeling and Sahm, 1980). This is however in contrast to other ob-servations using continuous cultures, which indicate that formaldehyde is also a good candidate to be the inducer (Zwart and Harder, 1983; Giuseppin et al., 1988a,b). For example, batch cultures of a MOX nega-tive mutant showed no induction of enzymes for methanol metabolism in the presence of methanol (Eggeling and Sahm, 1980), whereas in conti-nuous cultures both methanol and formaldehyde induce both MOX and the other enzymes for methanol metabolism to the same extent (Giuseppin et al., 1988a). However, whether methanol or formaldehyde is the actual inducer cannot be decided with the present data.

It has been found that in continuous cultures the enzymes involved in the methanol metabolism are induced in a coordinated fashion. The induction of the peroxisomal enzymes DHAS, MOX proceeds in parallel both

with respect to the appearance of specific mRNA and to the appearance of the enzyme activity (Egli et al., 1980; Egli et al., 1983; Giuseppin et al., 1988c). However, the cytosolic enzymes, formaldehyde dehydrogenase and formate dehydrogenase, are induced slightly more efficient as compared to MOX and DHAS. On the basis of the above observations the following working hypothesis can be formulated: the inducer interacts with a common protein (e.g. repressor) or protein complex that controls the synthesis of all the enzymes involved in the methanol metabolism. All these enzymes are, at least partially, under control of this common protein. Apart from this regulation, the absolute level of transcription may be modulated by additional genetic regulators such as the upstream activation sequences (UAS). Indications for the presence of UAS are given by the high degree of homology in the -1000 upstream region of both the MOX and DHAS structural gene of H. polymorpha (Unilever, 1986). However, more extensive genetic and physiological studies are needed to clarify the induction mechanism of MOX synthesis at the molecular level.

Repression of MOX synthesis.

During exponential growth in batch cultures of H. polymorpha on glucose, the synthesis of MOX and that of other enzymes involved in methanol assimilation, is repressed. This represents a long term metabolic regulation. Many other carbon sources repress the MOX synthesis as well, but the efficiency can vary considerably, ranging from 0-5 upto 70% of the induced state with methanol (Shimizu et al., 1977; Egli et al., 1983; Eggeling et al., 1980,1981; Koning et al., 1987), On the basis of data derived from both batch and continuous culture experiments, the following sequence of decreasing repression potential can be obtained:

ethanol >> glucose > sorbitol > glycerol, xylose > xylitol

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In batch cultures the effects of repression by glucose overrule the possible inducing effects of methanol or another inducing compound. In continuous cultures, with a relatively low concentration of residual glucose, however, this overruling effect is not observed. Under these cultivation conditions considerable levels of MOX activity may be present when mixtures of glucose and methanol are used as substrates. Depending on the dilution rate and the ratio of methanol to glucose, an optimal dilution rate with respect to MOX activity can be found (Egli, 1980; Giuseppin et al., 1988c). This indicates that at low dilution rates methanol has enough inducing potential to overrule glucose repression. At increasing dilution rates the concentrations of residual glucose increase. In that case repression induced by glucose is the counteracting factor, which results in a lower MOX activity (Giuseppin et al., 1988c; see also Chapter 2).

Degradation of MOX.

Apart from a reduction in the level of MOX by repression, its level in the cell can be negatively controlled by an inactivation process. When cultures with induced methanol metabolizing enzymes are transferred to a glucose containing medium, a rapid inactivation of MOX is observed (Veenhuis et al., 1983a). In spite of the high rate of inactivation this regulation may also be regarded as long term control of the methanol metabolism since the the inactivation rate is much slower (a half-time of about one hour) than the allosteric mechanisms. Furthermore the reversal of this process requires de novo synthesis of the enzyme, which is also a relatively slow process. The rapid inactivation involves a specific peroxisome directed autophagic process, which can be interpreted as a special form of catabolite inactivation induced by glucose. In general, catabolite inactivation in yeasts, a term proposed by Holzer (1976), is a series of reactions initiated by glucose or its metabolites leading to proteolytic breakdown of a specific protein. In contrast to this, the cytosolic dissimilatory enzymes formaldehyde dehydrogenase and formate dehydrogenase are not affected by this inactivation process.

Modelling of MOX synthesis.

The regulation mechanisms described show a complex system which is difficult to fit in a well-defined model. However, fermentation optimization studies require a mathematical model that describes the basic behaviour of the regulation mechanism in the long term. Therefore, a model was constructed on the basis of the two main controlling factors of the MOX synthesis, viz. repression and induction. For this purpose it can be assumed that methanol or formaldehyde forms a complex with a repressor molecule (Fig. 3), which is normally bound to the RNApolymerase binding site thus preventing transcription. The complex formation leads to transcription of the structural gene, i.e. induction.

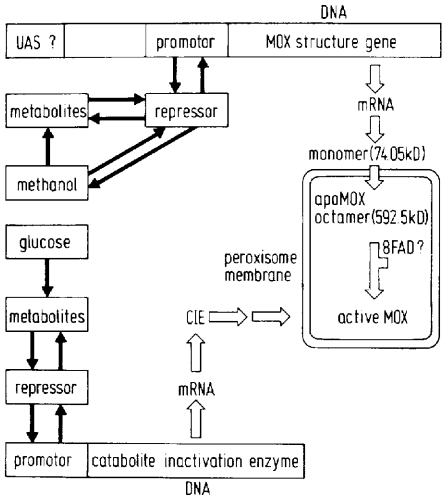

Fig. 3 : A working model for MOX synthesis and MOX breakdown in Hansenula polymorpha.

UAS, upstream activation sequence; MOX methanol oxidase; mRNA, messenger RNA; apo-MOX, Methanol oxidase without cofactor; FAD, flavine adenine dinucleotide; CIE, catabolite inactivation enzyme system.

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Repression of MOX is modelled on the basis of the following observations. It has been reported, that during growth of H. polymorpha in continuous cultures on methanol/glucose mixtures, high levels of MOXmRNA are formed up to high dilution rates (< 0.3 h^{-1}) i.e. induction. At high dilution rates (> 0.3 h^{-1}) lower levels of MOX-mRNA is found i.e. repression in the traditional sense. Under those cultivation conditions the formation of MOX-protein decreased dramatically above dilution rates of 0.14 h^{-1} (Giuseppin et al., 1988c). Furthermore, the MOX activity appeared to be very unstable at increasing dilution rates (Giuseppin et al., 1987), which may be caused by increased proteolytic activity in the yeast. For modeling purposes, this increased proteolytic activity is assumed to be induced by the increasing levels of residual glucose, which results in a type of catabolite inactivation. The induction of the enzyme system, which leads to catabolite inactivation (CIE), can be regarded as the main mechanism for the repression of MOX activity in continuous cultures. This process is called repression in this context. The presence of two systems, which lead to both synthesis and breakdown of MOX, seems rather inefficient for the cell. However, the imposed cultivation conditions are rarely found in nature and may therefore be regarded as an extreme condition for the cell, which results in an non-adequate response.

For the purpose of modeling the repression of MOX may be formulated as the induction of the catabolite inactivation system. Also for this induction process an equilibrium of glucose with the repressor of the hypothetical catabolite inactivation system is assumed (Fig. 3). The interactions of methanol and glucose with the repressor molecules can be described as a complex formation with a certain dissociation constant. They can be used to derive functions that describe the dependence of the efficiency of induction or repression on the concentration of methanol and glucose in the medium (Giuseppin et al,, 1988c; Chapter 6). These functions, "Q-functions", have been const-ructed and verified for many induction and repression phenomena by e. g. Yagil and Yagil (1971) and Toda (1976). The Q in these functions is the fraction of the maximal induction or repression level obtainable. The overall effective induction

fraction, Q, is the product of the Q-induction and Q-repression. In this way the induction level of MOX in continuous cultures grown on methanol/glucose mixtures can be modelled. At high dilution rates the high residual glucose concentration represses MOX synthesis (Qrepression decreases). At low dilution rates the inducing capacity of methanol will be the dominant effect (Q-induction). This model can describe the observed optimal dilution rate for MOX activity mentioned above. More information on the modelling of induction and repression is given in Chapter 6.

1.4 Biosynthesis of methanol oxidase.

The biosynthesis of proteins can generally be described using the sequence transciption of DNA into mRNA and the subsequent translation into protein also known as the central dogma of molecular biology. This sequence can be used to study the efficiency of the synthesis of a certain protein. For many enzyme production processes, it turned out that the synthesis of proteins may be dependent on factors such as the promotor efficiency, the transcription rate and the stability of the messenger RNA.

Apart from transcription and translation, the transport of the protein to the place in the cell for its action (protein topogenesis) may be important (e.g. Tabak, 1987). In case of peroxisomal enzymes, it is known that the synthesis of the protein occurs on free ribosomes in the cytoplasm. After that the protein is directed to the peroxisome.

This series of events also holds for MOX. The synthesis of MOX, however, is rather complex, because the enzyme is initially made as an inactive monomer in the cytosol (Bellion and Goodman, 1987), which is octamerized after transport into the peroxisomes (Goodman et al., 1984). On top of that also the incorporation of the cofactor FAD occurs in the peroxisomes. All these stages are summarized in Fig. 3. This scheme serves as a working model for the study of the efficiency of MOX synthesis in H. polymorpha.

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Transcription.

Until now, only some stages of the synthesis of MOX have been studied and little is known of the efficiency of the various steps involved. It was found that in batch cultures of H. polymorpha the synthesis of MOX Is largely determined by the level of MOX-mRNA (transcription stage) (Roggenkamp et al., 1984; Goodman et al., 1984). In these batch culture studies the organism was grown on either glucose or methanol, and thus only rough indications about the on and off mechanism of MOX synthesis could be obtained. The actual transcription or translation efficiency is not yet known, but there are strong indications that these efficiencies may depend on the cultivation conditions e.g. growth rate (Giuseppin et al. 1988c).

MOX monomers.

The next stage involves the synthesis of the MOX monomers. Under normal conditions these monomers are rapidly transported to the peroxisome and no or only low amounts of monomers are detectable (Roa et al., 1983; Giuseppin et al., 1988c). The routing of the monomers to the peroxisomes most probably occurs by means of an epitopic recognition site in the MOX monomer. No evidence has been found for alternative targeting in the form of a cleavable signal peptide or a pre-pro protein sequence (Ellis et al., 1985; Ledeboer et al., 1985). Comparative studies on the sequences of various peroxisomal proteins are now under way (W. Harder, personal communication) and will provide more evidence for such an epitopic recognition site.

The transport of the monomers into the peroxisomes is a largely unknown process. It has been shown for Candida boidinii that the actual transfer occurs via a complex of the MOX monomer with other proteins that so far have not been identified (Bellion and Goodman, 1987). However, there is no evidence for the occurence of such complexes in Hansenula polymorpha. The transport process may be dependent on the pH gradient across the peroxisome, which keeps the pH inside the peroxisome at 5.8 as compared to the value of 7.0 in the cytosol (Nicolay et al., 1987). This pH gradient is generated by a proton translocating ATP-ase in the peroxisomal membrane (Douma et al., 1987).

FAD incorporation in MOX.

During or directly after transport across the peroxisomal membrane, the monomers are octamerized and the cofactor FAD is incorporated. This octamerization and cofactor-binding may occur after a correct folding of the protein, which must occur after its passage through the membrane. Subsequently, the octamers are organized in a crystal. The FAD needed for MOX is supplied by a well regulated FAD biosynthetic route. This FAD synthesis is strongly increased in response to MOX synthesis (Shimizu et al., 1977a, b; Brooke et al., 1986). The experimental data on the growth on methanol clearly reveal that the rate of biosynthesis of FAD is sufficient for MOX synthesis. However, it is unknown whether this FAD biosynthesis is induced equally well using other growth conditions e.g. in case of high growth rates or when methanol/glucose mixtures are used as substrates. Furthermore, the efficiency of FAD incorporation may also depend on the growth conditions especially when mixtures of methanol with other carbon sources are used (Giuseppin et al., 1988c).

Activation of MOX.

It has been shown that MOX is not always present in the cell as an active enzyme (Veenhuis et al., 1976). Studies on methanol-grown cells, using electron microscopic techniques discriminating active from inactive MOX protein, showed that activation of MOX may occur at a certain stage of the cell cycle. Peroxisomes that have been transfered to a new daughter cell contain inactive MOX in a regular structure. This MOX protein is activated as soon as the cell separation is completed. Apart from that, it has frequently been observed that old cells contain peroxisomes with inactive MOX in regular structures. These data suggest a fast process of activation or inactivation, which is not yet known. Possible activation mechanisms may be based on specific phosphorylation reactions or on control of cofactor incorporation, which leave the crystal structure intact.

The brief outline of the biosynthesis of MOX shows that the many stages involved are still largely unknown with respect to their actual mechanism and efficiency for the active MOX formation. In view of the optimization of the MOX production it is essential to elucidate limiting stages in the biosynthesis of active MOX.

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1.5 Biochemical characteristics and purification of methanol oxidase.

For the potential application of MOX in detergents or other applications it is important to know the basic characteristics of the enzyme. In the past decade, methanol oxidases from various yeasts have been characterized biochemically (Table 1). From these data it appears that most types of MOX are FAD-containing homo-octamers with a molecular weight of about 600 kD. One exception is the MOX preparation derived from a P. pastoris strain, which was isolated in a tetrameric form of 300 kD. Generally said MOX contains one non-covalently bound FAD molecule per monomer. This seems to be the case for MOX preparations derived from cultures grown on methanol as the sole carbon source. The actual number of FAD molecules per octamer is probably not a constant. In that respect, it is remarkable that the reported figures range from seven to eight FAD molecules per octamer. This value depends on the amount of FAD and the estimate of the molecular weight of MOX. If the reported values are recalculated with the correct molecular weight of MOX (Ledeboer et al., 1985) the FAD contents will be considerably lower and in the range of six to seven FAD per octamer. A lower FAD level of about five to six has been found for MOX ex H. polymorpha grown on mixtures of methanol and glucose, which also indicates that the molar FAD/octamer ratio is not a constant and not as high as eight (Giuseppin et al. 1988c). These low figures may also reflect the effects of different cultivation conditions on the properties of MOX.

Specific activity of MOX,

The specific activities of the various MOX preparations can vary considerably (Table 1). It is remarkable that the specific activity for purified MOX preparations ex H. polymorpha may range from 5 to 15 Units/mg protein when grown on methanol, up to even 57.9 Units/mg protein. Here too, the cultivation condition may have large effects on the specific activity of MOX (Giuseppin et al., 1988c).

Table 1
Some basic biochemical properties of alcohol oxidases from various yeasts.

	Pichia pastoris				Hansenula polymorpha		Candida species		
Strain number	1	2	3	4	5	6	7	8	9
molecular weight (kD)	300	630	675	500	669	600	600	520	673
molecular weigh of monomer (kD)	76	75	80	72	83	74.05	74	65	84
number of subunits	4	8	8	>6	8	8	8	8	8
molar ratio FAD / MOX	-	-	7.3	>6	7.4	-	-	7.7	8.4
pH optimum for activity	-	6-8.5	7.5	8	8-9	8-10	7-10	6-8	8-9
Temperature optimum °C	-	40	-	45	45	50	30	37.5	35
Temperature stability *C **	-	38	-	-	50	-	-	30-60	43
Specific activity U/mg protein	-	12-30	-	-	15	15(57.9)	3.3	15	11

** At this temperature 50 % of the activity is lost after 10 min. - not reported.

One enzyme unit corresponds with 1 micromole substrate consumed per minute.
All the strains have been cultivated on methanol as the sole carbon source.

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Table 1 (ctd.)
Strain numbers : 1 Pichia pastoris NRRL Y 11328 (Patel et al., 1981); 2 Pichia pastoris NRRL Y 4290 (Hopkins and Muller, 1986); 3 Pichia pastoris IFF 206 (Couderc and Baratti, 1980); 4 Pichia pastoris NRRL Y 11430 (Pillips petrol Co., 1980,1982); 5 Hansenula polymorpha DL-1 (Kato et al., 1976); 6 Hansenula polymorpha CBS 4732 (Ledeboer et al., 1985; Veenhuis et al., 1983; van Dijken et al., 1976; van Dijken, 1976); 7 C. boidinii ATCC 32195 (Sahm and Wagner, 1973); 8 Candida 25-A (Yamada et al., 1979); 9 Kloeckera sp no 2201 (presently Candida)(Kato et al., 1976); 10 Torulopsis (presently Candida) R14 (Unichika, 1984) (not mentioned in Table 1).

The fraction of FAD involved in the catalytic cycle of MOX may also be an important factor.

Recent studies on MOX ex Candida boidinii suggest, that only about one third of the FAD present is involved in the catalytic cycle (Geissler et al., 1986). Although these studies were not carried out with MOX ex H. polymorpha, the observations may explain the large differences in specific activity found in terms of the catalytic efficiency of FAD in MOX. The various sources of MOX show a considerable variation of substrate specificity (Table 2). The differences between the various MOX types are especially evident for alcohols of longer chain lengths or substituted compounds as a substrate.

Affinity constant of MOX for substrates.

In the application of MOX at relatively low substrate concentrations (as needed in detergents) the affinity constant of MOX must be preferably low in order to allow addition of low concentration of enzyme and substrate. The affinity constants for some MOX types are given in Table 3. In general the affinity constant is lowest for methanol as substrate, but a considerable variation in the affinity constant is found for the various types of MOX. In most applications the affinity constant for the other substrate, oxygen, is an important factor as well. Especially in processes with low oxygen tensions or low oxygen transfer capacities, it is essential to model the two-substrate dependent kinetics (see also Section 1.2). Unfortunately only a few K-values and basic affinity

Table 2:

Relative activities of methanol oxidases on various substrates

	Pichia pastoris				Hansenula polymorpha		Candida species			
Strain number	1	2	3	4	5	6	7	8	9	10
Substrate										
methanol	100	100	100	100	100	100	100	100	100	100
formaldehyde	15	33	-	-	13	-	-	23	55	-
ethanol	92	36	82	100	50	70	75	82	106	35
n-propanol	74	20	43	73	44	-	25	38	79	60
2-propanol	0	2	2	4	-	-	5	21	-	-
allyl alcohol	-	30	-	-	-	-	65	-	-	-
n-butanol	52	10	20	45	32	-	15	27	69	53
n-pentanol	30	-	-	5	-	-	-	21	-	-
2-mercapto ethanol	25	7	-	-	-	-	-	-	-	-
2-chloro ethanol	70	10	-	-	38	-	-	-	71	-
alcohol concentration	0.1[a]	25[b]	0.93[a]	20[b]	sat.	100[a]	5[a]	5.7[a]	100[b]	sat.
oxygen concentration (mM)	0.21	0.26	0.93	0.27	0.24	0.21	0.24	0.24	0.24	0.24
temperature °C	37	25	37	23	30	37	30	30	30	30

See Table 1 for explanation of the strain numbers; sat.: measured under conditions of saturation of the alcohol; - : not reported; a: concentration in mM; b: concentration in $mg \cdot l^{-1}$.

Table 3:

Affinity constants of yeast alcohol oxidases

	Pichia pastoris			Hansenula polymorpha		Candida species	
Strain number	1	3	4	5	6	8	9
Substrate							
methanol	0.5	1.4	4	0.23	1.3	0.019	0.44
methanol with excess oxygen	-	3.1	-	-	2.8 **	-	-
oxygen with excess methanol	-	1	-	-	0.4 **	-	-
formaldehyde	3.5	-	3.5	2.6	-	-	2.4
ethanol	-	-	4.4	-	-	0.13	2.5
1-propanol	-	-	14	-	-	-	5.7
1-butanol	-		40	-	-	-	9.1
2-chloro ethanol	-		12	-	-	-	21

The affinity constants are expressed as mM at air saturation except for values on row two and three.

- : not reported; **: the corresponding dissociation constant K is equal to 0.13 mM; See Table 1
for explanation of the strain numbers.

constants are reported in literature, which makes it difficult to compare the MOX preparations by modelling of the reaction kinetics.

Stability of MOX.

All applications of MOX require a high stability of the enzyme during the process. Unfortunately, the enzyme is inactivated by the reaction

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products formaldehyde and hydrogen peroxide (Geissler et al., 1986). Formaldehyde inactivates MOX only at high concentrations of about 0.4 to 1 M (Sakai and Tani, 1986, 1987). This inactivation is also found when methanol grown cells are exposed to an excess pulse of methanol (Veenhuis et al., 1980). The inactivation of MOX by hydrogen peroxide follows a Michaelis-Menten type of reaction kinetics. The inactivation parameters reported for MOX ex Candida boidinii are 1.6 mM and 33 h^{-1} for K,H_20_2 and the maximal inactivation rate, respectively. MOX preparations ex Pichia pastoris and H. polymorpha show a lower hydro-genperoxide sensitivity, characterized by higher K, H_20_2 values (> 8 mM) and lower maximal inactivation rates (< 1 h^{-1}) (Giuseppin et al., 1988b, Hopkins and Muller, 1987). This product-inactivation limits the application of MOX in conversion processes, in which a high product concentration is needed. In crude preparations the formation of hydrogen peroxide has less dramatic consequences, because it is efficiently decomposed by catalase.

Down stream processing of MOX.

For most applications MOX must be recovered and purified to a certain extent to remove undesired contaminants. A general scheme for the recovery of MOX from H. polymorpha is given in Fig. 4. The first stage consists of a centrifugation to harvest the cells and to remove unwanted medium components. After this stage a cell-disruption is needed to recover the soluble intracellular protein. Finally this cell-free extract has to be purified. Even though MOX is present in levels up to 40% of the cellular protein, purification is necessary because of high levels of undesirable catalase activity. Catalase is an enzyme with a high molar turnover number of 5 to 6 x 10^6 enzyme cycles per minute. This high specific activity requires rigorous procedures to purify MOX (Bruinenberg et al., 1982; Phillips Petroleum Co., 1980) or to inactivate catalase (Verduyn et al., 1984; Unilever, 1983). Although these methods result in rather pure MOX preparations with low levels of catalase activity, they are expensive. Furthermore, the methods employing chemical inactivation of catalase may lead to unwanted traces of toxic

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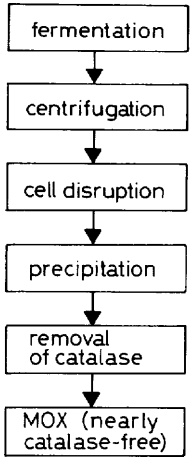

Fig. 4 : Process steps for the production of MOX.

compounds in the final product. An attractive alternative to make catalase-free MOX preparations is to make use of a catalase-negative mutant of H. polymorpha (this thesis Chapter 3). By using such a strain the need to use expensive purification methods can be circumvented.

1.6 Optimization of the process for methanol oxidase production.

In most cases the application of enzymes is limited by the relatively high production costs. These high costs can be devided into fixed costs such as investment cost, and variable costs such as substrate and processing costs a discussion of these costs, however, is beyond the scope of this thesis.) This necessitates a good optimization of the production process after the identification of a suitable enzyme and/or a production organism.

The main problem in optimizing fermentation processes is the choice of the method. Apart from that, many boundary conditions of the optimizations depend strongly on the large scale facilities and techniques of the available production plant. These boundary conditions limit the freedom to optimize the process. In general, however, the optimization must fulfil the criterion of high overall productivity (Skøt, 1983) and high efficiency in terms of money-in versus money-out per unit of time, which is common in the design of chemical industry. These criteria are used for the whole plant and on segments or unit operations in that plant. However, in contrast to optimization of unit operations in chemical processes, the interactions of the various unit operations in an enzyme production process are often not known or difficult to predict. For example, using microorganisms there are many interrelated variables, such as the growth rate and medium composition that may have large effects on the cell wall strenght or the enzyme content of the cell, which may influence other stages in the production process. This implies that a straightforeward optimization of the unit operation fermentation and other unit operations will not necessarily lead to an optimal overall process. The unit operations involved in the production process for MOX are given in Fig. 4. They can be regarded as stages: the fermentation and the down stream processing stage.

Optimization criterion.

The boundary conditions for the optimization of the process considered in this thesis are formed by the yeast H. polymorpha, the enzyme MOX and the type of fermentation. A continuous fermentation process is chosen. Firstly, it has been shown that high yields of MOX can be obtained in continuous cultures. Secondly, continuous cultures enable systematic studies on the physiology and fermentation, which can also be used to design other types of processes e.g. fed-batch processes. The optimization criterion for both the fermentation and the down stream processing is formulated as the optimization of the specific productivity (P/V) of a given continuous culture (expressed as amount of active recoverable MOX produced per liter fermenter volume per hour).

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A simple formula can be used to describe this P/V criterion:

$$P/V = D \cdot X \cdot E \cdot S \cdot R$$

This formula may be used as a guideline for the optimization of the process. Furthermore the formula provides a simple tool to discuss the optimization with researchers from other disciplines. The variables in the formula can be studied individually or in relation to other variables. The variables are: D, dilution rate (h^{-1}); the biomass concentration, X, in gram dry weight cells per liter; E, the expression level of the MOX gene and the transcription efficiency; S, the stability of the MOX-gene product.

This latter parameter includes the efficiency of translation into protein, post-translation and the processes leading to the final formation of active MOX; R describes the overall efficiency of the down stream processing in terms of the fraction of MOX that can be recovered. It must be noted that the P/V-formula does not include various cost factors such as investments and material costs.

Term D·E·S.

The factors E and S are strongly dependent on the cultivation conditions. The induction and repression mechanisms, described in Section 1.3, are included in the values of E and S. Expression and stability are of course functions of the dilution rate. These dependencies lead to optimization of the terms D·E, D·S or combined D·E·S. The study of these terms includes the determination of E, e.g. MOX-mRNA level, and the stability of MOX at various dilution rates (Giuseppin et al., 1988c). The term E·S, which implies a high specific activity in terms of enzyme units per gram biomass (or protein) ($U \cdot gX^{-1}$), can also be interpreted as a high specific activity of MOX in terms of enzyme units per gram MOXprotein. Many physiologically and genetically determined aspects of the optimization procedure are covered by E·S.

Physical limitations.

Some variables are limited by physical factors rather than biological factors. For example, the biomass concentration, X. The obvious limit of X determined by the volume of the yeast cells. This corresponds with pressed baker's yeast, with a concentration of about 250 g dry weight·l^{-1}. Especially at large scale operation the biomass concentration may be limited by the oxygen transfer rate (OTR, $gO_2 \cdot l^{-1} \cdot h^{-1}$), the heat transfer rate (HTR, $J \cdot l^{-1} \cdot h^{-1}$). Oxygen transfer limitation will lead to a maximally obtainable biomass concentration, Xmax, given by the formula:

$Xmax \leq OTR,max / q0_2$.

In this formula the $q0_2$ stands for the specific oxygen consumption rate ($902 \cdot gbiomass\text{-} \cdot l^{-1} \cdot h^{-1}$), which is a function of the dilution rate and biological parameters, according to the linear relationship:

$q0_2 = D / Yox + mo$.

In this formula the biological parameters for yield on oxygen, Yox (g biomass·$g0_2^{-1}$), and the maintenance oxygen consumption, mo ($g0_2 \cdot$ gbiomass·h^{-1}, are important. The actual values will depend on the type of carbon source used (Giuseppin et al., 1988c, Roels, 1983) and must be determined for the conditions used. From this example it can be concluded that the maximum of X depends on D as well. This leads to a frequently used optimization criterion with the term X·D (Skøt, 1983). In those cases in which the oxygen transfer limitations can be overcome, the heat transfer, strongly correlated with the OTR, may be the limiting factor. This will often occur at large scale operations. The HTR is related to the OTR via the empirical relation that 1 mol of oxygen consumed by the microorganism yields 455 kJ of energy in the form of heat (Roels, 1983). The above mentioned formulas to calculate the maximal obtainable biomass concentrations give only theoretical estimates. The actual maximal values are lower and depend on the degree of homogeneity of substrate and biomass in the reactor.

Using continuous cultivation techniques, the reported maximal biomass concentrations are considerable. In this way about 133 g/l dry Pichia pastoris can be produced commercially at a dilution rate of 0.10 -0.14 h^{-1} (Phillips petroleum Co., 1983, 1984a, b).

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Recovery of MOX.

Another important factor in the P/V formula is the recovery term, R. This term covers the effects of growth conditions on the cell break-up and the purification procedure needed. It has been shown in the literature that the growth rate greatly influences the cell wall properties such as thickness and resistance to physical and enzymatic treatments (Baratti et al., 1978; Bruinenberg et al., 1985; Christi and Moo-Young, 1986; Giuseppin et al., 1987). At increasing growth rates the cell wall becomes more sensitive to the disruption procedures. When less rigorous procedures can be used, the inactivation of MOX may decrease, resulting in a higher MOX yield. The yield of the purification can also be improved by using a catalase-negative strain of H. polymorpha (this thesis Section 1.5 and Chapter 3). For some applications the recovery term be can almost neglected, especially when whole (dried) cells are used. Freeze-dried cells for example may be used as a detergent ingredient (Unilever, 1987a).

Reliability of the fermentation process.

It is important to have a reliable and robust fermentation process besides the criterion of a high P/V. Especially large-scale continuous cultivation requires a good knowledge on the factors that influence the reliability of this expensive process.

The reliability of the process can be lowered by a poor dynamic stability of the culture against process disturbances. Continuous cultures of H. polymorpha grown on methanol may be very sensitive to sudden pH shocks (Swartz, 1978), changes in oxygen tension (Dudina, 1984; Swartz and Cooney, 1981), disturbances in methanol supply rate and local low oxygen tensions as a result of poor mixing. During or after these disturbances, the cultures may accumulate inhibiting or even toxic levels of formaldehyde and formate via overflow of the metabolic routes described in Fig. 2 (Pilat and Prokop, 1976a, b; Giuseppin, 1988d). The resulting substrate and product inhibited growth kinetics of the culture has many implications for the stability of the culture at certain dilution rates, and even for the start-up procedure of a continuous culture with high cell densities. When no adequate measures are taken under those circumstances, the culture may be washed-out due to a low growth rate, or lethal concentrations of the compounds methanol, formaldehyde or formate. In

many cases the main problems with culture stability in continuous cultures can be overcome by using mixtures of methanol with other carbon sources.

The robustness and reliability of any microbiological process, particularly continuous cultivation, relies heavily on the way of hygienic and aseptic processing. It is relatively easy to maintain asepsis in continuous fermentation on laboratory scale, but it is difficult to operate large scale continuous fermentations absolutely aseptically for a long time. To enhance the reliability in this respect, the medium composition and the cultivation conditions may be adapted to improve the intrinsic stability of the medium against infections. This can be done by lowering the pH of the medium-feed to pH 2 or by fermenting at a lower pH of 3-4 (Phillips Petroleum Co., 1983).

1.7 Outline of this thesis.

The factors that may determine the productivity of the production process for methanol oxidase have been studied in order to optimize the process. The productivity of the fermentation and down-stream processing (P/V) has been described using a simple formula, which accounts for the individual variables in a continuous process. P/V has been defined as the product of the dilution rate, D, the biomass concentration, X, the expression of the MOX gene, E, the stability of the gene product, S, and the recovery yield in the down-stream processing, R. These variables have been studied separately and in relation to other variables using continuous cultures.

The bottlenecks in the stages of the biosynthesis of active MOX, described by the variables D, E and S, have been studied in continuous cultures of H. polymorpha grown on a methanol/glucose mixture. Chapter 2 shows the results of these studies, which include the effects of the dilution rate on the levels of specific MOX-mRNA and MOX-protein, and on the cofactor (FAD) content of MOX.

In order to optimize the recovery, R, a route to circumvent expensive recovery methods has been tested. In this case, alternative ways to induce MOX in a catalase-negative mutant of H. polymorpha has been studied. This has lead to the use of formaldehyde/glucose and formate/glucose mixtures as described in Chapter 3.

Although methanol is known to be toxic for catalase-negative strains, studies have been undertaken to use methanol/glucose mixtures to induce MOX. This study was also aimed at the elucidation of the physiological implications of the absence of catalase (Chapter 4).
The recovery of MOX by cell break-up using physical or biochemical means, may strongly depend on the fermentation conditions used. The effects of dilution rate and type of substrate on the cell wall strenght is given in Chapter 5.
A mathematical working model describing growth and MOX production is needed for the optimization of the production process. A first attempt to model the complex phenomena is given in Chapter 6.

The findings described in Chapter 3 and 4 have resulted in two patent applications. In Chapter 7 a summary of these patents is given.

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Chapter 2

MOLECULAR REGULATION OF METHANOL OXIDASE ACTIVITY IN CONTINUOUS CULTURES OF HANSENULA POLYMORPHA

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SUMMARY

The regulation of methanol oxidase (MOX) in Hansenula polymorpha has been studied in continuous cultures using a mixture of glucose/ methanol (4:1 w/w) as carbon source. The study focused on the identification of stages in the biosynthesis affecting the formation of active MOX in glucose/methanol grown-cells. The levels of MOX mRNA, MOX protein in monomeric and octameric form, the ratio FAD/ MOX, and the actual MOX activity have been quantified as functions of the dilution rate (D). Hybridisation studies with MOX mRNA probes showed an induction of MOX mRNA formation upto D = 0.29 h^{-1}. The induction of MOX protein synthesis (upto 37% of the cellular protein) is determined at low D-values on the transcriptional level. MOX activity at high D-values is tuned by FAD incorporation and (post-)translation. Despite the high levels of MOX mRNA, decreasing levels of MOX activity and MOX protein were found at D-values ranging from 0.14 to 0.29 h^{-1}. The maximal ratio FAD/MOX (6) was determined at D = 0.1 h^{-1}, which correlated with the maximal specific activity of MOX. In glucose/methanol media both protein level and MOX activity are repressed by increasing levels of residual glucose at high D-values.

INTRODUCTION

Methanoloxidase (MOX) plays an important role in the physiology of methylotropic yeasts. It occurs as a homo-octamer in crystalline structures in Hansenula, Pichia and Candida species in organelles called peroxisomes[1]. The role of MOX in the physiology of Hansenula is well understood.

The regulation of MOX and the related physiology of methylotrophic yeasts have been studied extensively in the past decade[2]. However, the production of MOX has not been studied as a process, and its optimization has not been described yet.

The induction of MOX can be accomplished using methanol[3,4] or mixed substrates[5,6] such as glucose/methanol mixtures. The factors governing

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the regulation of MOX-activity in H. polymorpha have not been determined completely. Besides indications of direct genetic control of MOX synthesis, an active proteolysis directed to MOX is found in cultures having high residual glucose concentrations[7]. The relation between MOX-mRNA level and MOX activity is not known. High levels of MOX may be caused by correspondingly high levels of MOX-mRNA. A specific probe of MOX-mRNA is needed to determine this correlation.

Little and contradictory information is available on the steps determining the formation of active MOX in the peroxisomes. Veenhuis et al.[1] found indications that the ultimate formation of active MOX limits MOX activity in continuous cultures grown on methanol. They found nonactive MOX containing peroxisomes during cell budding until cell wall separation occurred. Although the incorporation of the co-factor FAD is essential in MOX activation, there are no data in the literature indicating at which stage FAD is incorporated.

In this paper we will present results of a study on the factors that possibly affect or limit the formation of active MOX in H. polymorpha grown on glucose/methanol mixtures in continuous cultures. The following factors have therefore been studied as functions of the dilution rate (D):

- MOX-mRNA levels to quantify the transcription to mRNA and its correlation to active MOX;
- the possible accumulation of (inactive) monomers of MOX at high D-values;
- the ratio FAD/MOX octamer, since FAD is essential for active MOX;
- the actual amount of active and inactive MOX octamer produced per total soluble, cellular protein.

Identification of the limiting factor in the formation of active MOX is essential for optimization of the MOX production process to enable industrial application.

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MATERIALS AND METHODS

Organism and growth conditions

Strain. Hansenula polymorpha CBS 4732 (wild-type strain).

Media. As developed by Egli[6] except that the antifoaming agent PPG was omitted from the medium. They were sterilized at 120°C for 20 min. Vitamins and methanol were sterilized separately by filter sterilization. The total inlet concentration of substrate (glucose/ methanol) was kept at 10 $g \cdot l^{-1}$ (unless stated otherwise).

Cultivation. H. polymorpha was cultivated in continuous cultures using a Chemoferm fermenter (Chemoferm, Sweden) with a working volume of 1.5 to 2.5 l. The temperature was kept at 37 ± 0.2°C; the pH was kept at pH 5.0 ± 0.05 by addition of an ammonia solution (35% w/w) containing the antifoam Rhodorsil R426 in a ratio of 4:1 (v/v). The medium feed was introduced together with the air supply using one nozzle to improve the substrate mixing in the culture, especially at low D-values. The oxygen tension was kept above 25% saturation. Steady states were determined by measuring the respiration parameters and the MOX activity levels in whole-cell suspensions and in the cell lysate.

Lysis

Procedure. 200 ml Cell suspensions with an optical density (OD) of 15 to 18 (5.5 to 6.6 g dry cells$\cdot l^{-1}$) were made in a solution containing 0.1 M sodium phosphate buffer pH 8.5, 5 mM EDTA, 1 mM dithiotreitol and 10 mg zymolyase 100000 ex Arthrobacter luteus (Seikagaku Kogyo Co., Japan). The solution was stirred gently in a thermostatted vial. Every 20 min, samples were taken and analysed for MOX activity, protein content and optical density at 610 nm. The high-purity zymolyase was used to avoid possible proteolytic activity from the enzyme preparation during cell lysis (Giuseppin et al.8). Ultrasonic treatments were carried out with a Branson cell disruptor (70 W, with microtip); 5-ml-aliquots of wet

cell mass and glass beads (ratio 1:1, Ø glass beads 100-150 µm). The lysis was carried out in five treatments of 1 min with cooling periods in between the sonifications. The disruption by glass beads was performed in a Virtis homogenizes during 5 min in 0.01 M Tris/HC1 buffer (pH 8).

Assays

Gas analysis, The exhaust gases were analysed for $C0_2$ with a UNOR 6N and for 0_2 with the MAGNOS 2T (both Hartmann & Braun). The level of dissolved oxygen was determined with an Ingold autoclavable polarographic electrode. Respiration characteristics such as oxygen uptake rate, carbon dioxide evolution rate and static oxygen transfer rate were calculated on-line with a MINC 11/23 minicomputer.

The MOX activity in cell lysates and HPLC fractions was determined according to van Dijken et al.[3]. The MOX activity is expressed as pmol methanol consumed per minute with 40 mM methanol as substrate; in cell suspensions 80 mM methanol was used.

Glucose and methanol were determined enzymatically[9]. Methanol concentrations < 10 mg/kg were also determined by GLC.

Dry weight levels were determined after drying a washed cell suspension at 110°C for 16 h. Estimations of biomass in biochemical assays were made by determining the optical density (OD) at 610 nm in 1-cm cuvettes; the values were converted into dry weight with a calibration curve.

The protein level of the lysed cells was determined according to Lowry et al.[10], with bovine serum albumin as standard.

MOX content. MOX was analysed quantitatively by FPLC. The deep-frozen cells were resuspended in 50 mM Tris/HCl, pH 8.0. The cells were broken in a Virtis 45 homogenizes (Virtis Company; Gardiner NY, USA) using glass beads of 250-300 Mm for 10 min at 0°C. The supernatant was passed through a YM 30 filter (Amicon) with a molecular mass cut-off of 30 kDa. The clear supernatant was brought onto a Mono Q column (Pharmacia, Sweden) and eluted with 50 mM Tris/HCl buffer, pH 8.0, at a flow rate of 1 ml/min. A linear gradient of 0 to 1 M NaCl was used to obtain optimal

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separation of MOX from the other proteins. 0.5 ml fractions were collected and analysed for MOX, catalase and protein.

FAD contents. The extinctions at 280 and 438 nm were measured in the above fractions. FAD contents were calculated according to Couderc and Baratti[11] in native MOX protein and heat-denatured MOX protein. The molar extinction coefficient of FAD under the experimental conditions was 11.3 x 106 $cm^2 \cdot mol^{-1}$ at 450 nm. For the calculation of FAD contents, a molecular mass of 592.5 kDa (based on DNA sequence data) was assumed for the protein without FAD[12].

mRNA levels of MOX and DHAS. RNA was isolated from cells kept in liquid nitrogen as follows: 0.25 g cells were suspended in 2 ml buffer that contained 25 mM sodium phosphate, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM mercapto ethanol, 100 units/ml zymolyase and 2 M sorbitol pH 7.5. After incubation at 30°C for 5 min the cells were spun down and resuspended in 1.5 ml phenol solution previously saturated with 1 M Tris and 0.1% 8-hydroxyquinoline. To this mixture 1.5 ml STE solution (1 M NaCl, 0.1 M Iris pH 7.6, 0.01 M EDTA, and 1% SDS) was added. After centrifugation, the waterphase was extracted once more with 1 volume of phenol solution and 0.5 volume of CIA (chloroform and iso-amylalalcohol in a ratio of 24:1). The DNA and RNA were precipitated from the waterphase by adding 0.1 volume of 0.1 M sodium acetate pH 5.8 and 3.0 volumes of ethanol. After storage overnight at -20°C and centrifugation, the RNA was precipitated by adding 1 volume of 8 M LiCl. The precipitate was resuspended in STE (containing 0.1% SDS). The concentration of RNA was determined by measuring the absorption at 260 nn. The RNA was stored as an alcohol precipitate at -20°C.

Blotting,-prehybridisation and hybridisation Blotting was carried out according to Thomasl[3]. Gelelectrophoresis was performed on 1% agarose, 16,% formaldehyde gel pH 7.5 with 15 µg RNA. After prehybri-disation, the hybridisation was carried out with 50 ml hybridisation mixture[13] plus 0.15 µg probe; MOX specific: $4.2 \cdot 10^8$ cpm/µg, DAS specific: $3.1 \cdot 10^8$ cpm/µg). This mixture was incubated at 42°C for 16 h in a shaking waterbath. The blots were washed three times for 15 min with a solution containing 0.75 M NaCl and 0.085 M trisodium citrate at room temperature, Two more 15-min-washes were done at 45°C using a dilution of 2.5 of the above mixture (though still with SDS 1 g/1). The amount of probe left on

the blots was determined by exposing the films at -80°C for 6 to 16 h. The blackening of the exposed films was quantified by densitometric measurement. A grey scale on the film of 10 to 90% transmission was compared with the blank.

Probes. The 31-mer probe used for MOX mRNA was complementary to the MOX gene sequence
5'-GCAGCAGCCGGTGGAACCTCCACCAACAACA-3.
The DHAS probe, a 32-mer, had the sequence
5'-GCTCGACAATGTCCAGGACAAGAGCAGGACCG-3'. According to CG content, the two probes had the same binding strength. 0.3 µg of the probes were labelled with ^{32}P (^{32}P-ATP 2000 cpm/fig) using 2,6 MBq[14,15]. The amount of sample was standardized to a constant amount of ribosomal RNA.

Gelelectroghoresis of the protein samples - to show the MOX monomers - was performed in the presence of 0.1% SDS using 7.5% polyacryl-amide gels. The amount of protein applied was adjusted to constant total protein quantities for all lanes. The level of MOX monomers in the cell lysates was quantified by visual inspection of and densitometric measurement on the coomassie blue-stained gels.

RESULTS AND DISCUSSION

Growth of H. polymorpha on different carbon sources

Steady-state values for Ysx from several separate continuous culture runs are given in Fig. 1. The yield coefficients, calculated according to Lee et al.[16], have been summarised in Table 1. The values are similar to those found by e.g. Egli et al.[17], and van Dijken et al.[3].

$Y_{sx\ max}$ in case of glucose/methanol (4:1 w/w) is slightly lower than that found by Egli et al.[6, 17]: 14.6 against 15.2 gX·mol C^{-1}. $Y_{sx\ max}$ on methanol is slightly higher than that found by the above workers: 14.7 against 12.2-13.4 gX·mol C^{-1}. The maintenance substrate consumption rate (m_s) is low for glucose/methanol 4:1; in case of glucose/methanol 1:1, however, m_s is close to that found with methanol only,

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Table 1. Growth parameters for H. polymorpha as a function of the carbon source. Yield coefficients and maintenance terms have been calculated on the basis of electron and carbon balance data according to Lee et al. (1984).

Parameter		methanol	glucose/methanol 1:1	glucose/methanol 4:1
Y_{sx}	[gX·mol C^{-1}]	14.7	12.4	14.6
Y_{ox}	[gX·mol 0_2^{-1}]	25.0	28.9	40.5
m_s	[mmol C·gX^{-1}.h^{-1}]	0.78	1.0	0.05
m_o	[mmol 0_2·gX^{-1}·h^{-1}]	2.1	2.5	0.38
μ_{max}	[h^{-1}]	0.21(0.18)*	0.2	0.52(0.51)*
MOX	yield (units·mmol C^{-1}]	27.0	31.6	31.0

* washout

The cultures grown on glucose/methanol 1:1 (w/w), in contrast with those grown on glucose/methanol 4:1 (w/w), or methanol only, are extremely unstable to process disturbances, especially at D-values higher than 0.2 h^{-1}. Disturbances such as air bubbles in the medium feed or a sudden drop in pH of 5.0 to 4.5 caused the culture to be killed within a few seconds. Despite repeated attempts to obtain steady states at D >0.2 h^{-1} in the 1:1 mixture, it was impossible to collect data. These disturbances have not been described by others; they seem to be characteristic of cultures grown on glucose/methanol 1:1.

No relevant parameter e.g. the accumulation of methanol-derived metabolites, formaldehyde, formate or medium deficiencies could be identified as a possible cause for the above phenomenon, since it was too fast to be explained by these factors. A highly speculative explanation is that of the inbalance of the glycolyse/gluconeogenese at this particular ratio of glucose/methanol and high growth rates. The metabolic pathways as proposed by Egli et al.[2] have in principle a built-in futile cycle (an ATP-spilling reaction) at the branchpoint of glucose and methanol assimilation, consisting of fructose1,6bisphosphase and phosphofructokinase. Disturbances

may lead to a fatal loss of ATP. Further studies are required to clarify this phenomenon.

MOX protein and activity levels as a function of D

MOX activity. In continuous cultures the MOX activity levels were determined for glucose/methanol ratios of 4:1 and 1:1; methanol being used as reference. The activities were determined using a zymolyase-mediated lysis procedure, which proved to give reproducible enzyme recoveries[8]. The specific activities, expressed as the maximal MOX activity recovered per g dry biomass, are given in Fig. 1.

Electrophoretic analysis of MOX. A polyacrylamide gel electrophoresis (PAGE) with lysates of cells taken at several steady-states is shown in Fig. 2. This figure clearly shows the disappearance of the two forms of MOX protein. The MOX band on SDS-PAGE represents total MOX protein in its monomer form (molecular mass 74 kDa). There is a considerable overlap with the DHAS band (78 kDa). The intensity of the MOX band on SDS-PAGE decreases at D-values > 0.19 h^{-1} parallel to the decrease in MOX activity found in lysates (Results of Western blotting confirm this. Data not shown.). No large amounts of inactive monomers seem to be formed at high D-values. At D =0.35 h^{-1} only very low amounts of MOX protein are found on PAGE, which is in agreement with the analytical data given below.

FPLC analysis. Active and inactive octamer levels were determined using a 0 to 1 M NaCl gradient elution on Mono Q FPLC. A typical example of the well-defined separation of MOX protein and activity from other proteins is shown in Fig. 3. A summary of the results is given in Fig. 4. The specific activity of the MOX protein peak found in the lysates has a clear optimum at D = 0.1 h^{-1}. At D = 0.05 h^{-1} a low specific activity is found, correlated with a low FAD content of the MOX peak.

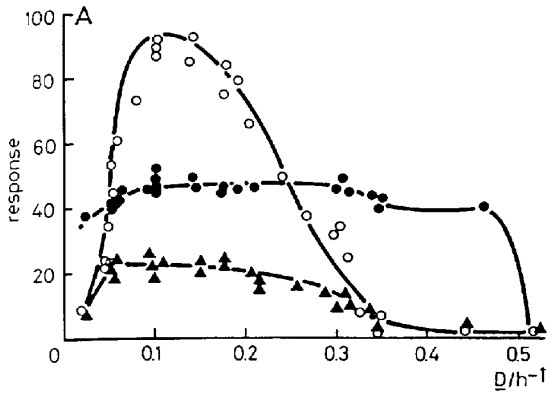

Fig. 1A. Steady-state values of MOX activity and cell yield in H. polymorpha. Substrate: glucose/methanol 4:1 (w/w).
● ysx [(gX·gS^{-1})·100); O activity [(MOX-units·gX^{-1})·0.04];
△ MOX "in vivo" [(µmol 0_2·gX^{-1}·min^{-1})·0.04]

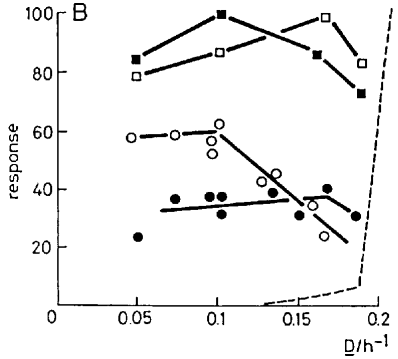

Fig. 1B. Steady-state values of MOX activity and cell yield and MOX and DHAS-mRNA levels in H. polymorpha, Substrate: glucose/methanol 1:1 (W/W).
□ MOX mRNA (%); ■ DHAS mRNA (%); O MOX activity [(MOX-units·gX^{-1}) · 0.02]; ● Ysx [(gX·gS^{-1})·100); - - - methanol concentration (mg/l)·0.1

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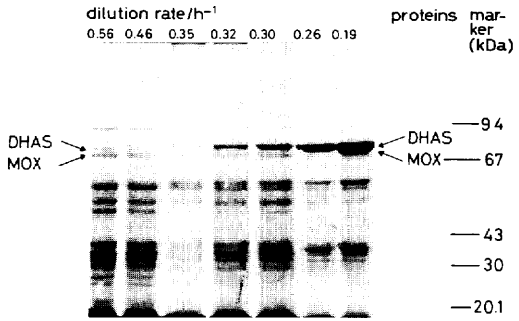

Fig. 2. PAGE analysis of MOX at several dilution rates. Substrate: glucose/methanol 4:1 (w/w).

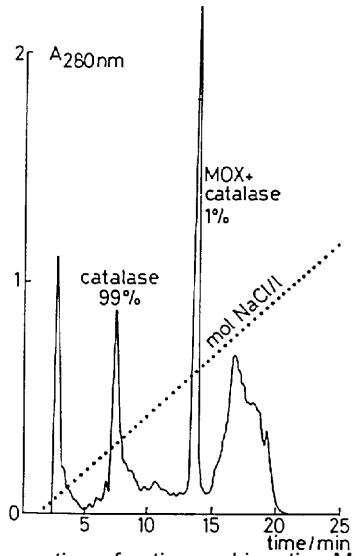

Fig. 3. Separation of active and inactive MOX octamer (using a 0 to 1 M NaCl gradient solution on Mono Q FPLC).

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The MOX protein levels in the lysates in the D-range of 0.05 to 0.19 h^{-1}, however, remain constant at a level of 36-37% of the soluble protein with a molecular mass ≥ 30 kDa.

The protein recovery yield for ultrasonically treated cells, zymolyase - treated cells and glass-bead milled cells have been compared. The various methods give the same specific activities for MOX in the lysate.

Physiological MOX activity. The change in MOX activity in cell-free extracts at various D-values has little effect on the maximal in vivo methanol conversion rate of whole-cell suspensions (Fig. 1). The maximal oxygen consumption rate of whole-cell suspensions after a methanol pulse shows a maximum value of 0.75 μmol 0_2·(mg X·min)$^{-1}$, whereas the MOX activity in cell-free extracts can be more than four times this value.

Similar differences were found by van Dijken et al.[3]. A diffusion limitation of methanol and oxygen across the cell membrane and - to a lesser extent - the peroxisomes cause a lower maximal uptake rate of whole-cell suspensions. The diffusion limitations across the membrane can be temporarily overcome by permeabilizing the cell wall with e.g. Chitosan[4], resulting in response of the cells to methanol comparable to that of a cell-free extract. The maximal methanol conversion rate, calculated from the maximal oxygen consumption rate, yields an upper limit of 2.9 g methanol·g X^{-1}·h^{-1}, which is much higher than the maximal steady-state methanol consumption rate of 0.4 to 0.45 g methanol·gX^{-1}·h^{-1} as reported by Egli et al.[17]. From these values it is evident that MOX activity is not growth-rate limiting[2].

FAD content of MOX

The decrease in MOX activity at D-values < 0.1 and > 0.2 h^{-1} may be caused by a lack of activated MOX octamers. A nearly constant FAD/MOX protein ratio of 5-6 has been found, with a maximal ratio at D = 0.1 h^{-1}. At D = 0.05 h^{-1}, a significantly lower FAD content is found at a constant MOX protein level. At D-values > 0.1 h^{-1} a slight decrease of FAD content was observed. At D = 0.46 h^{-1}, FAD could not be determined very accurately because of its low concentration in the FPLC fractions.

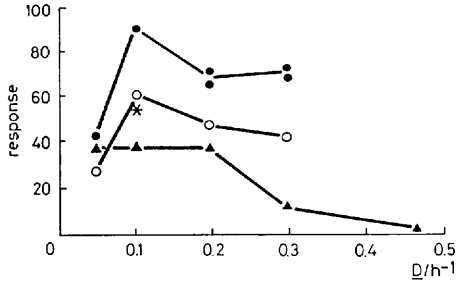

Fig. 4. FPLC analysis of MOX at several dilution rates. Units have been corrected for catalase activity. Molecular weight: 592.5 kDa. Substrate: glucose/methanol 4:1 (w/w). ▲ MOX protein (%)·10; ● MOX activity [(MOXunit·mg protein^{-1})·10]; O FAD/MOX (mot/mol)·10; ✶ reference FAD/MOX ratio with methanol as substrate

The maximal FAD/MOX ratio (Fig. 4) is significantly lower than the value of 7.5 to 8 as reported by Kato[18]. When this ratio, which is based on a monomer mol mass of 83 kDa, is corrected with the recently found mol mass of 74.05 kDa[11] a ratio of 6.6 is found. The protein levels determined by FPLC correspond well with the semiquantitative PAGE protein patterns (Fig. 2).

The biosynthesis of FAD may be the limiting factor at high D-values. Some experiments have therefore been carried out to study the effects of the addition of riboflavin, the precursor of FAD; H. polymorpha was cultivated at D-values of 0.05 and 0.3 h^{-1} in the presence of 0.1 mM riboflavin. The Ysx and specific MOX activity did not increase upon addition of riboflavin. Mechanisms other than the limitation in FAD synthesis seem to be involved.

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mRNA levels of MOX

The methanol-assimilative enzyme system is assumed to be concertedly induced by methanol and the MOX synthesis is probably limited on the transcriptional levellg. Experiments in batch cultures only show a positive correlation of MOX synthesis and the corresponding occurrence of mRNA. In order to obtain more quantitative data on the rate-limiting steps of MOX synthesis, the mRNA level of MOX was determined.

mRNA was isolated from steady-state samples of several continuous culture runs using glucose/methanol 4:1 as limiting carbon source. There is a linear relationship between RNA level (% w/w, dry weight) and D; a reference sample of a methanol-grown culture agrees well with these data (Fig. 5). This linear relationship between D and RNA level is usually found for bacterial RNA levels, and the small standard deviation in the RNA levels indicate that the isolation of RNA is equally efficient for the various growth rates. At wash-out conditions (maximal μ) and D = 0.56 h^{-1}, the upper limit of 3.7% RNA (w/w, dry weight; precipitable with LiCl) is obtained.

In cultures of H. polymorpha grown on glucose/methanol 4:1 (w/w), the relative level of MOX-mRNA increases with increasing D (Fig. 6); this increase of mRNA correlates with MOX formation and activity. For comparison, a specific probe for dihydroxyacetone synthase (DHAS) mRNA was used. DHAS is a key enzyme, situated in the peroxisomes, which converts formaldehyde into dihydroxyacetone and enables the assimilation of formaldehyde formed by the MOX-mediated reaction. The pattern for DHAS mRNA formation is similar to that of MOX mRNA formation. This is in agreement with the concerted induction of MOX and DHAS activity at various glucose/methanol ratios as reported by other workers[2, 20]. 6 to 7% of the total mRNA is MOX-mRNA and another 6 to 7% is DHAS-mRNA (Z.A. Janowicz, personal communication). The D-values showing high mRNA levels do not correspond with the optimum D levels for MOX protein and MOX activity. Furthermore the mRNA levels of MOX mRNA do not decrease parallel with the decrease of MOX activity or MOX protein. These findings support the hypothesis that MOX synthesis is regulated by

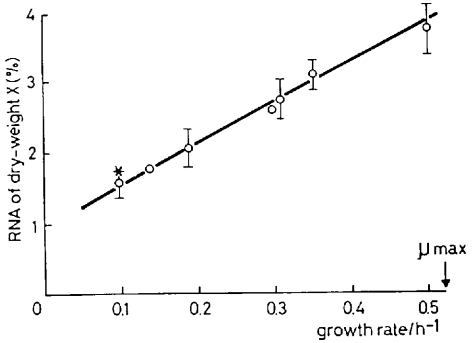

Fig. 5. RNA levels of H. polymorpha as a function of growth rate.
Isolation by LiCl-precipitation. Substrate: glucose/methanol 4:1 (w/w).
★ reference with methanol as substrate

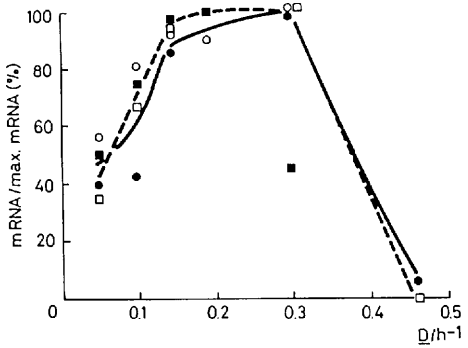

Fig. 6. Relative mRNA-levels of MOXand DHAS in H. polymorpha as a function of dilution rate, D. Substrate: glucose/methanol 4:1 (w/w).
□ - - □ and ■ - - ■ DHAS-mRNA; O—O and ●—● MOX-mRNA;
□ O - after LiCl-precipitation, ■ ● - in crude extract

an induction process and that the activity of MOX at high D-values is limited by factors other than those at the DNA-transcription level.
The results of cells grown on glucose/methanol 1:1 have been compared with those for glucose/methanol 4:1. mRNA levels in case of glucose/methanol 1:1 (Fig. 1) show a slightly different pattern for MOX and DHAS mRNA. The MOX-mRNA level is optimal at D=0.17 h^{-1} (near the critical D-value). The DAS-mRNA level has its optimum between D=0.1 and 0.17 h^{-1}. The higher residual concentration of methanol in glucose/methanol 1:1 allows formation of mRNAs at lower D-values than that of glucose/methanol 4:1 does.

CONCLUSIONS

The growth of H. polymorpha on glucose/methanol mixtures yields a culture with a high MOX content and an activity up to 37% of the soluble cellular protein content and a molecular mass >30 kDa. The specific MOX activity is comparable with that of methanol-grown cultures at D-values of 0.05 to 0.1 h^{-1}. The yield of MOX - expressed as MOX units·(mmol C) $^{-1}$ is constant in the carbon sources used: approximately 28 - 31 MOX units·(mmol C)$^{-1}$.

The formation of active MOX may be limited by many steps in the synthesis of the active octamer, depending on the growth condition applied. At low D-values, the active MOX level is determined by FAD incorporation. The mRNA level, constant protein level and low FAD/ MOX protein ratio give strong evidence for this assumption. At D = 0.1 h^{-1}, an optimal FAD content of 6 FAD per octamer MOX is found, having a constant protein and increased MOX-mRNA content. At D-values > 0.25 h^{-1}, the FAD content slightly decreases simultaneously with both octameric and monomeric MOX protein. The mRNA level of MOX, however, does not decrease when the MOX activity does, showing that the transcription is not rate-limiting. As no significant pool of MOX monomer could be detected at D > 0.25 h^{-1}, the efficiency of MOX formation is most likely determined by the translation of MOX-mRNA or the decomposition of MOX monomer by glucose-induced proteolysis in the cytoplasm.

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NOMENCLATURE

D	dilution rate	[h^{-1}]
DHAS	dihydroxyacetone synthase	
FAD	flavin adenine dinucleotide	
MOX	methanol oxidase	
ms	Maintenance substrate consumption	[mol C·gX^{-1}·h^{-1}]
x	biomass	
Ysx max	maximum yield of biomass on substrate	[gX·mol C^{-1}]
Yox max	maximum yield of biomass on oxygen	[gX·mol 0_2^{-1}]
μ_{max}	maximum growth rate	[h^{-1}]

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Chapter 3

PRODUCTION OF CATALASE_FREE METHNOL OXIDASE BY HANSENULA POLYMOPHA

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Summary. Many of the potential technical applications of alcohol oxidase (MOX; EC 1.1.3.13) are limited by the presence of high activities of catalase in the enzyme preparations. In order to circumvent laborious and costly purification or inactivation procedures, the induction of MOX in a catalase-negative mutant of Hansenula polymorpha has been studied. Emphasis was laid on the induction of activities of MOX and the dissimilatory enzymes in continuous cultures grown on various mixtures of formate/glucose and formaldehyde/glucose. In continuous cultures of the catalase-negative mutant grown on these mixtures, MOX can be induced efficiently. To obtain a stable and productive process, the ratio of the substrates is of critical importance. The optimal ratios of the mixtures for the catalase-negative strain for formate/glucose and formaldehyde / glucose were 3:1 and 1-2:1, respectively. Under identical cultivation conditions the wild-type strain showed similar induction patterns for MOX and the dissimilatory enzymes formaldehyde dehydrogenase (FaDH) and formate dehydrogenase (FoDH). The MOX levels in the catalase-negative strain were approx. 58% of those in the wild-type strain.

Introduction

Methanol oxidase (MOX) can be induced in methylotrophic yeasts in amounts ranging from 20 to 37% of the soluble cellular protein (van Dijken et al. 1976, Giuseppin et al. 1988, Veenhuis et al. 1983). This enzyme may have potential technological applications in the generation of hydrogen peroxide for bleaching processes (Unilever 1984), in the colorimetric determination of alcohols (Verduyn et al, 1984), and in the production of aldehydes such as formaldehyde (Tani et al. 1985). The application of MOX as a hydrogen peroxide producer, however, depends on the quantitative removal of catalase, as this enzyme decomposes the hydrogen peroxide formed.

Catalase can be removed from the MOX preparations by separation (e.g. Patel et al. 1981) or chemical inactivation (Verduyn et al. 1984). Unfortunately, these methods are costly and difficult to scale up, and the latter may introduce toxic compounds in the final MOX preparation.

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A catalase-negative strain of Hansenula polymorpha would offer an attractive alternative for the production of catalase-free MOX. Though this strain grows well on glycerol or glucose (Eggeling and Sahm 1980), it cannot grow on methanol as the sole carbon source probably because of the toxic effects of hydrogen peroxide formed by the MOX-mediated reaction. In batch cultures, growing on glucose or glycerol, MOX is produced under conditions of derepression, at the end of the exponential growth phase, which leads to a low specific MOX activity of approx. 17% of that found in cultures of the wild-type strain; this level is too low to make the production of catalase-free MOX economically feasible.

MOX can also be induced in glucose grown batch cultures of Candida boidinii (Eggeling et al. 1977) and Kloekera sp. no 2201 (Shimuzu et al. 1977a and b) by adding formate or formaldehyde to the culture. In this case, the cultures use formaldehyde and formate as energy source only and do not assimilate these compounds. Although formaldehyde and formate show an induction effect in batch cultures, Eggeling and Sahm (1980) have concluded that methanol rather than its metabolites is the inducer for MOX. Therefore our study focused on the induction of MOX in both a wild-type strain and in a catalase-negative mutant derived from H. polymorpha (CBS 4732) in continuous cultures using glucose as a carbon source.

Materials and methods

Organisms and growth conditions

Strains. Hansenula polymorpha CBS 4732 (wild-type), and a catalasenegative mutant of H. polymorpha, ATCC 46059 derived from CBS 4732. Other differences are not known.
Media. As described by Egli (1980). Formate was added either as sodium salt or as formic acid. Methanol-free formaldehyde was prepared by hydrolysing paraformaldehyde in 0.1 M sodium hydroxide for 2 h. The glucose concentration in the feed was 10 $g \cdot l^{-1}$.

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Cultivation: The strains were cultivated in continuous cultures using a fermenter with a working volume of 2 1. The pH was maintained at pH 5.0 ± 0.05 with a mixture of antifoaming agent, (silicon oil, Rhodorsil 426 R, Rhone Poulenc) and concentrated ammonia. The temperature was maintained at 37°C. The oxygen tension was always higher than 25%. Samples from steady states were obtained after four to five residence times. Steady-states we also checked by determining MOX activity, carbonere dioxide production and oxygen consumption rate. They were constant for at least one volume change.

Preparation of cell-free extracts

The cell-free extracts were prepared by treating the cells ultrasonically (Branson cell disruptor B12) maintaining a power input of 70 W per 5 ml solution. The cell suspension of 0.5 g wet cells and 3 g glass beads (mean diameter 100 gym) was treated five times for one minute with one-minute intervals at 0°C.

Assays

Activities of MOX. catalase. formaldehyde dehydrogenase (FaDH) and formate dehydrogenase (FoDH) in cell-free extracts were determined according to van Dijken et al. (1976). One MOX-unit corresponds to one umol methanol consumed per min at 37°C in an air-saturated 0.1 M potassium phosphate buffer at pH 7.5. Catalase-units are expressed as Δ E 240 nm per min. All other units are expressed as pmol substrate converted per min.
The protein level was determined according to Lowry et al. (1951). Bovine serum albumin was used as standard.
The biomass level was determined by drying a washed cell suspension at 110°C for 16 h.
Metabolites. Glucose, methanol, formic acid and formaldehyde were determined using HPLC (Aminex HPX 87H. Biorad; column temperature 60°C, eluent 0.005 M H2S04, flow 0.8 $ml \cdot min^{-1}$, detection by a differential refractometer) and enzymatic assays according to: verduyn et al. (1984), methanol; Nash (1953), formaldehyde; Lang and Lang (1972), formate.

The protein composition of the cell free extracts was determined by FPLC using a MONO Q column (Pharmacia). The column was eluted with a buffer (50 mM TRIS/HC1, pH 8.0) using a 0 to 1 M NaCl gradient (Giuseppin et al. 1988). MOX and catalase were determined in all fractions. The protein in the fractions was determined using the absorption at 280 rim, and as described by Lowry et al. (1951). The FAD content was determined according to Giuseppin et al. (1988).

Results and discussion

Effect of formate/glucose mixtures on growth and MOX production

The catalase-negative strain was grown in continuous cultures (dilution rate D = 0.1 h^{-1}) on mixtures of formate/glucose at molar ratios in the range of 0 to 6.6. Fn all cases stable steady states were obtained.

The wild-type strain was also grown under similar conditions for comparison. MOX in catalase-negative H. polymorpha (Fig. la) was induced efficiently up to a maximum level of 58% of that in the wild-type (Fig. lb) under similar conditions. The optimal formate/glucose ratio is 2.9. Higher ratios (4-6.6) show a plateau of the levels of MOX and the dissimilatory enzymes. (Data not shown).

FoDH was clearly induced at the lowest formate/glucose ratios (< 1.4). In the wild-type strain, the FoDH activity increases from 0 to 0.4 U·mg protein^{-1} at a molar ratio of 2.6. The catalase-negative strain shows a similar FoDH activity at a ratio of 3.6. The reported maximal FoDH activity in H. polymorpha grown on methanol and methanol/glucose mixtures has been reported to be 0.1-0.4 U·mg protein^{-1} (van Dijken 1976, Egli 1980). The maximal FoDH activity observed of 0.4 U·mg protein^{-1} corresponds with a maximal formate consumption rate of 10 mmol formate·h^{-1}·g biomass^{-1}, assuming 40% of the biomass to be pro-tein. This FoDH activity is similar to that found for Pichia pinus grown on

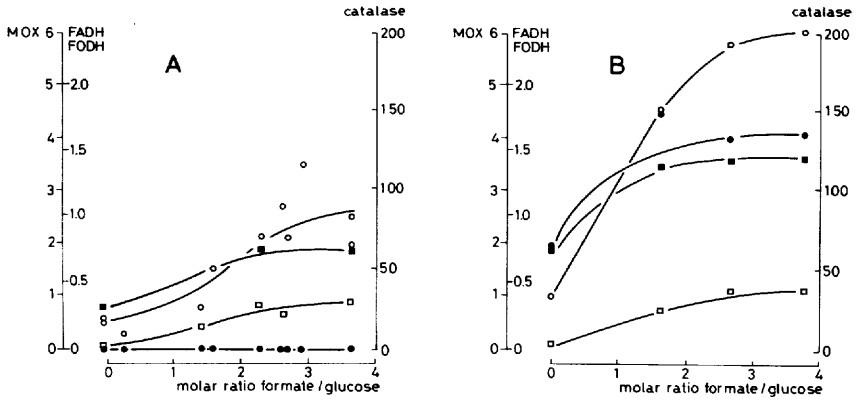

Fig. 1 Enzyme patterns (in U·mg protein^{-1}) in continuous cultures of Hansenula polymorpha, catalase-negative strain (A) and wild-type strain (B) grown on various formate/glucose mixtures
MOX (O); catalase (●); FoDH (□); FaDH (■).

formate/methanol mixtures (Muller et al. 1986). The formate consumption rate in the chemostat at the highest formate/glucose ratio was about 6.8 mmol formate·h^{-1}·g biomass^{-1}. A slight overcapacity for formate consumption is available under these conditions. This value is close to that reported for the methanol consumption rate for steady-state cultures grown on methanol / glucose mixtures: 12-14 mmol methanol·h^{-1}·g biomass^{-1} (Egli et al. 1986).

The increase in catalase activity of 64 up to 150 U·mg protein^{-1} in the wild-type strain clearly shows the induction of MOX activity correlated to catalase besides a basal catalase activity. This catalase activity is needed to destroy the hydrogen peroxide formed in the peroxisomes, if the organism is grown on methanol.

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Effect of formaldehyde/glucose mixtures on growth and MOX production

The catalase-negative and wild-type strains were grown in continuous cultures (D = 0.1 h^{-1}) using ratios of formaldehyde/ glucose in the range of 0-1.8. The steady-state values of the MOX levels (Fig. 2) clearly show that formaldehyde is a better inducer of MOX than formate. The optimal formaldehyde/glucose ratio was about 1.4 for the wild-type strain; that of the catalase-negative strain showed an increase upto the highest ratio tested. The concentration of residual glucose in the substrates was below the detection limit of the assays (< 1 mg·l^{-1}), that of residual formaldehyde was below 3 mg·l^{-1}.

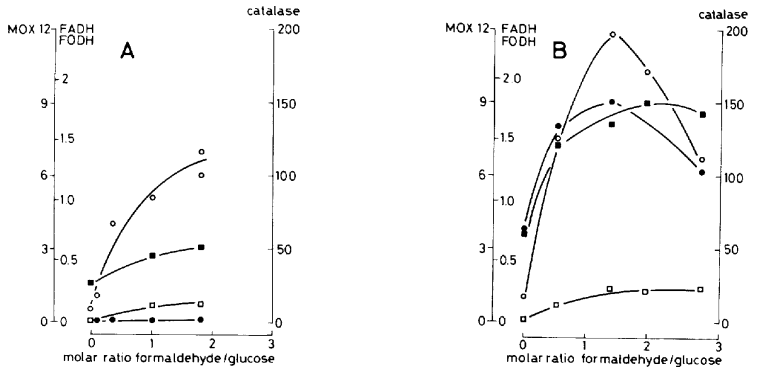

Fig. 2 Enzyme patterns (in U·mg $protein^{-1}$) in continuous cultures of
A. catalase-negative and B. wild-type Hansenula polymorpha grown on various formaldehyde/glucose mixtures
MOX (O); catalase (●); FoDH (□); FaDH (■).

At high formaldehyde/glucose ratios (≥ 2.2) the formaldehyde level becomes toxic (> 0.3 mM), which inhibits growth and causes a wash-out of the culture. The maximal FaDH activity, in cells grown methanol and methanol/glucose mixtures, reported is 0.7-1.3 U·mg $protein^{-1}$ (van Dijken et al. 1975, Egli 1980). The maximum FaDH activity observed in the wild-type strain (1.7 U·mg $protein^{-1}$) is significantly higher than that in the catalase-negative strain (0.67 U·mg $protein^{-1}$). These differences in FaDH activity may reflect unknown physiological responses to the absence of catalase or unknown genetic differences between the wild-type and catalase-negative strain.
Because of the difference in FaDH activity between wild-type and catalase-negative strain, the maximal formaldehyde dissimilation rate, and consequently the maximal dilution rate and maximal molar ratio, at which steady-state growth is possible, will differ considerably. The maximal flux through FaDH in the cell in steady-state cultures, based on the "in vitro" FaDH activity, will be 19 and 43 mmol formaldehyde·h^{-1}·g $biomass^{-1}$ for the catalase-negative and wild-type strain, respectively. The maximal formaldehyde uptake rate observed was about 2 mmol formaldehyde·h^{-1} ·g $biomass^{-1}$ for both the catalase-negative and the wild-type strain. This low rate may indicate toxic effects of the formaldehyde itself or its oxidation product, formate, in the cell.

Effect of dilution rate on growth of and MOX production by the catalase negative mutant

The effect of D on MOX production was studied in a medium using a formate/glucose ratio of 3.6 (Fig. 3). At D = 0.24 h^{-1}, the formate concentration increases significantly with a decrease of recoverable MOX.

The biomass yield on glucose remained constant within the range of the dilution rates tested. The decrease in MOX activity is similar to the decrease caused by glucose repression as found in continuous cultures of H. polymorpha grown on methanol/glucose mixtures at various dilution rates (Egli 1980, Giuseppin et al. 1988). The repression caused

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by the increasing residual glucose concentrations at increasing dilution rates may explain this low MOX activity in continuous cultures grown on formate/glucose mixtures. D-values in the range of 0.05 to 0.15 h^{-1} are optimal for the ratio of formate/glucose applied.

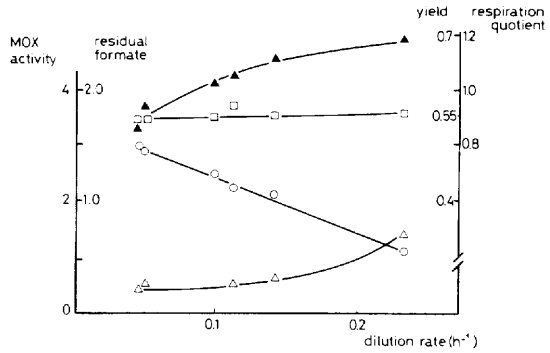

Fig. 3 Effect of dilution rate on MOX production by catalase-negative Hansenula polymorpha. Mixture: formate/glucose 3.6 : 1.
MOX activity, U·mg protein $^{-1}$ (O); residual formic acid, mM (△);
respiration quotient, mol $C0_2$/mol 0_2 (▲); yield, g biomass/g glucose (□).

Protein composition

The HPLC chromatograms of MOX preparations from both catalase-negative and wild-type H. polymorpha are given in Fig. 4. The peak, eluted after 8 min, containing catalase activity, is not present in preparations of the catalase-negative strain. Catalase activity normally associated with MOX in the wild-type strain, was not found.

The (cofactor) FAD content is about 5 mol FAD·mol MOX^{-1}, which is close to the values of 5-6 found for the wild-type strain as reported by Giuseppin et al. (1988).

The catalase-negative strain, the culture and MOX properties were stable for more than 2000 h in continuous cultures at D = 0.1 h^{-1}.

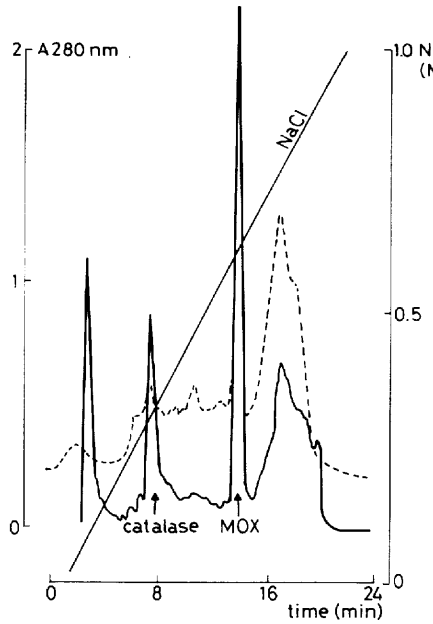

Fig. 4
FPLC elution patterns of cell-free extracts of catalase-negative and wild-type Hansenula polymorpha.
___ wild-type; - - catalase-negative.

Conditions catalase negative strain: mixture formate/glucose 2:1, dilution rate 0.1 h^{-1}.
Conditions wild-type strain: mixture glucose/methanol 4: 1, dilution rate 0.19 h^{-1}. MOX peak contains 1% of the total catalase activity.

Conclusions and general discussion

MOX can be induced in continuous cultures of both the catalasenegative and the wild-type strain of H. polymorpha using the oxidation products of methanol. Efficient induction is obtained in continuous cultures using media with formate/glucose and formaldehyde/glucose. The molar ratio of the substrates has been shown to be of critical importance for a good MOX yield. When formaldehyde is used the stability of the culture is strongly dependent on the dilution rate and molar ratio employed.
The induction of dissimilatory enzymes such as FoDH and FaDH is considerable in both formaldehyde/glucose and formate/glucose. FaDH is

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induced equally well by formaldehyde and formate. The induction of FaDH in formate/glucose-grown cultures - apparently not requiring FaDH - indicates that the induction of FaDH and FoDH by formate is concerted. However, the level of FoDH induction by formate is higher than that by formaldehyde.

The level of MOX in the catalase-negative strain grown at D = 0.1 h^{-1}, under glucose limitation is 60% of that in the wild-type strain. Under identical conditions, the MOX levels in the catalase-negative strain are 40% and 55% as compared to those in the wild-type strain for formate/glucose and formaldehyde/glucose, respectively. This system-atically lower MOX activity indicates that the catalase-negative strain has a catalase-negative mutation and other mutations e.g. in the MOX promoter, causing a lower transcription efficiency. In addition also FaDH is expressed at a system-atically lower level. This may indicate a diffe-rence in the action or availability of one regulator molecule, which is involved in the induction of both MOX and FaDH.

The continuous cultivation and induction methods enable significant improvement of the productivity per fermenter volume compared to the batch wise MOX production under derepressed conditions as described by Eggeling and Sahm (1980). The continuous fermenter can produce more than 300 MOX units·g biomass^{-1}·h^{-1}, whereas in batch production no more than 7.7 MOX units·g biomass^{-1}·h^{-1} can be obtained (using a fermentation cycle time of 48 h).

The method described in this paper can lead to a less expensive production process of MOX for use on a large scale. The omission of expensive steps -purification, or catalase inactivation - gives the general production scheme as described in Fig. 5. For commercial applications no further purification is needed, and dried whole cells can be used for the generation of H_20_2 or for other applications of MOX (Unilever 1986).

Although methanol is regarded as the actual inducer of the C-1 metabolic pathway (Eggeling et al. 1977), more studies are needed to explain the high inducing capacity of formaldehyde and formate. Some evidence of induction of C-1-assimilative enzyme systems for growth on

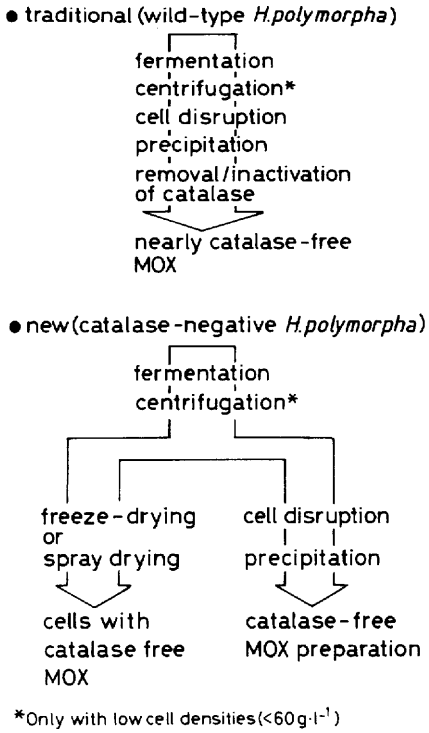

Fig. 5 MOX production process scheme for wild-type and catalase-negative Hansenula polymorpha.

methanol and methylamine by the key compound formaldehyde was found for H. polymorpha by Zwart and Harder (1983). Whether this induction was caused by intracellular conversion of the compounds into methanol or by stereochemical similarities of the hydrated form of these compounds is still unknown.

Acknowledgement

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Chapter 4

UTILIZATION OF METHANOL BY A CATALASE -NEGATIVE MUTANT OF HANSENULA POLYMORPHA

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Summary. In methanol-utilizing yeasts, catalase is an essential enzyme for the destruction of hydrogen peroxide generated by methanol oxidase (E.C. 1.1.3.13). It was found however that a catalase-negative mutant of Hansenula polymorpha is able to consume methanol in the presence of glucose in continuous cultures. At a dilution rate of 0.1 h^{-1}, stable continuous cultures could be obtained during growth on methanol/glucose mixtures with a molar ratio of methanol/glucose between 0 to 2.4. In these cultures methanol oxidase was induced up to a level of 40% of that obtained in the wild-type strain. The hydrogen peroxide-decomposition activity of the mutant was studied in more detail by pulsing methanol to samples of steady-state cultures. Only after the addition of excess methanol the hydrogen peroxide-decomposing system became saturated, and the cells excreted hydrogen peroxide. This was accompanied by excretion of formaldehyde and a rapid loss of viability. The presence of extracellular catalase during a methanol pulse prevented the loss of viability. The nature of the alternative hydrogen peroxide-decomposing enzyme system remains to be elucidated. Its capacity strongly depended on the cultivation conditions and pretreatment of the cells. Cells grown on formaldehyde/glucose mixtures showed a lower methanol tolerance than those grown on the methanol/glucose mixtures. Freeze-drying of cells drastically enhanced the excretion of hydrogen peroxide, probably as a result of an inactivation of the decomposing system.

Introduction

Methanol oxidase (MOX) has many potential applications, e.g. the generation of hydrogen peroxide (Unilever patents 1984, 1986) and the production of primary aldehydes (Tarsi et al. 1985). The feasibility of the first application, from an economic point of view, depends on the complete removal of contaminating catalase activity. This removal step can be circumvented by inducing MOX in continuous cultures of a catalase-negative strain of Hansenula polymorpha grown on mixtures of formaldehyde/glucose and formate/glucose (Giuseppin et al. 1988). The catalase-negative strain is reported not to grow on methanol, probably because of a MOX-mediated accumulation of toxic levels of hydrogen peroxide in the

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cell (Eggeling and Sahm 1980). It was assumed that media containing a substrate of MOX would kill the culture immediately.

We have tested the tolerance of the catalase-negative mutant to methanol. The organism was grown in continuous cultures on a mixture of formaldehyde/glucose with a high level of MOX activity as described recently (Giuseppin et al. 1988). Contrary to expectation it was found that after a pulse of methanol to a cell suspension only small amounts of hydrogen peroxide were formed. Furthermore, the hydrogen peroxide production occured only after a considerable lag phase. These observations led to the hypothesis that the catalase-negative mutant possessed an alternative hydrogen peroxide-destruction system. This would also imply that the mutant can utilize methanol to a certain degree. In this paper we will describe studies on the capacity of this alternative hydrogen peroxide-destruction system and the utilization of methanol by this strain grown in continuous cultures on methanol/glucose mixtures.

MATERIALS AND METHODS

Strains

Hansenula polymorpha ATCC 46059, a catalase negative mutant derived from the wild-type strain CBS 4732, was used in this study (Eggeling and Sahm 1980).

Media

The media used were as described by Egli (1980) and Giuseppin et al. (1988). The initial glucose concentration was kept at 5 g·l-1. Methanol was added separately after filtration.

Cultivation

The mutant was cultivated in continuous cultures using a fermenter with a working volume of 2 l (Chemoferm,Sweden). The pH was maintained at 5.0 ± 0.05 with 12.5% ammonia. The temperature was maintained at 37 ± 0.1°C. The dissolved oxygen tension was kept above 25% air saturation. To control foaming, an anti-foaming agent, silicone oil (Rhodorsil 426 R, Rhone Poulenc) was used in a concentration of 0.01% in the medium. Samples from steady states were taken after four to five dilutions, after which the MOX activities, carbon dioxide production and oxygen consumption rate were constant. Continuous cultures grown on methanol/glucose mixtures were started up as a continuous culture grown on glucose at the same dilution rate (D). The methanol concentration in the feed was gradually increased, while keeping the residual methanol concentration at a low level (< 20 mg $\cdot l^{-1}$).

Preparation of cultures for methanol pulse experiments

Steady-state continuous cultures on formaldehyde/glucose or methanol /glucose mixture were used. The molar ratio of the mixture was 1 and D was kept at 0.1 h^{-1}. The glucose concentration in the feed was 5 $g \cdot l^{-1}$. Samples of the steady-state cultures (100 to 175 ml) were quickly transferred to a fermenter of 250 ml and kept under the same conditions as in the continuous culture. After a 5-min incubation period, methanol was injected to the concentration indicated in the text. The dissolved oxygen tension was kept above 75 % air-saturation.

Determination of affinity of whole cells for methanol

The rate of methanol-dependent oxygen consumption by whole cell suspensions was determined in a biological oxygen monitor (Yellow Springs Instruments Co., USA) at 37°C using an air-saturated buffer (0.05 M potassium phosphate, pH 7.5). Catalase (ex bovine liver, 3 x 10^5 $U \cdot l^{-1}$) was added to the buffer to protect the cells against the hydrogen peroxide. The affinity constant was determined using a Hanes plot. The rate of methanol-dependent oxygen consumption was calculated assuming a stoichiometry of 1/2 mol 0_2 consumed per 1 mol methanol oxidised.

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Preparation of cell-free extracts

The cell free extracts were prepared by treating the cells ultrasonically (Branson cell disruptor B12) with a power input of 70 W per 5 ml solution. A cell suspension of about 0.12 g wet cells was made in a 0.1 M sodium phosphate buffer pH 7.5. The cell suspension of 0.5 g wet cells and 3 g glass beads (mean diameter 100-150 gym) was treated five times for 1 min (with one-minute intervals) at 0°C.

Enzyme assays

The activities of MOX, formaldehyde dehydrogenase (FaDH) and formate dehydrogenase (FoDH) and catalase were determined according to van Dijken et al. (1976). One MOX unit corresponds with 1 μmol of methanol consumed at 37°C in an air-saturated 0.1 M potassium phosphate buffer at pH 7.5. Catalase activity is expressed as decrease in absorbance at 240 nm per min (van Dijken et al. 1976). All other enzyme units are expressed as μmol substrate consumed per minute. Glutathione peroxi-dase was determined using the method of Paglia and Valentine (1967) and by consumption of hydrogen peroxide in the presence of reduced glutathione. The viability of the cells was determined by quickly plating out the cell suspensions on YEPD agar, which consisted of 2% yeast extract, 1% peptone and 2% glucose. The lates were incubated at 37°C for 2 days before counting the colonies.

Analytical methods

Protein. Protein was determined according to Lowry et al. (1951). Bovine serum albumin was used as a standard.

Biomass. Biomass was determined after drying a washed cell suspension at 110°C for 16 h.

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Glucose. methanol, formic acid and formaldehyde were determined using HPLC (Aminex HPX, Biorad) with a refractory meter and standard enzymatic assays according to Verduyn et al. (1984), Nash (1953) and Lang and Lang (1972) for respectively methanol, formaldehyde and formate.

Hydrogen peroxide was determined using an iodine reaction in an auto-analyser (the final reaction mixture contained 8 $g \cdot l^{-1}$ KI, 1 $g \cdot l^{-1}$ $(NH_4)_2$ $Mo0_4$, and 1 $mol \cdot l^{-1}$ H_2S0_4). Low H_20_2 concentrations (<34 $mg \cdot l^{1}$) were measured using a reagent containing horseradish peroxidase (10 $U \cdot l^{-1}$) and 2,2'-azino-di(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS; 10 $mg \cdot l^{-1}$). This reagent gives a green color at 630 nm. The detection limit was about 50 $\mu g \cdot l^{-1}$.

Chemicals.

Methanol-free formaldehyde was prepared by dissolving paraform-aldehyde in 2 M sodium hydroxide at 60°C. ABTS, horse radish per-oxidase and catalase were obtained from Sigma (USA).

Results

Growth and enz ym production on methanol/glucose mixtures

From preliminary experiments it was concluded that the catalase-negative mutant might be able to tolerate methanol or, to a certain level, even grow on media containing methanol as an additional carbon source. Indeed this proved to be the case. The catalase-negative mutant could be adapted to growth in continuous cultures on methanol/glucose mixtures. In these cultures both methanol and glucose were consumed quantitatively. Accumulation of hydrogen peroxide could not be detected as its concentration was below the detection limit of 50 $\mu g \cdot l^{-1}$. The culture showed stable fermentation characteristics such as respiration rate and constant enzyme levels of MOX, FaDH and FoDH. The catalase-negative mutation was stable for more than 2000 h of continuous cultivation.

The MOX activity in the catalase-negative strain was induced maximally at a molar ratio of methanol/glucose between 1 and 2 (Fig. 1). This

is close to the optimal range of methanol/glucose ratios of 1 - 1.4 as found by Egli (1980) and Egli et al. (1982). Molar ratios higher than 1.8 caused the specific MOX activity to decrease, which is in contrast to the constant activity of MOX in cultures of the wild type (Egli 1980). The maximal level of specific MOX activity of 4 U·mg protein, however, is about 40 to 50% of that of the wild-type strain under similar conditions (Egli 1980). The optimal molar ratio of methanol/glucose is similar to that of cultures of the catalase-negative mutant growing on formaldehyde / glucose mixtures (Giuseppin et al. 1988), suggesting a similar induction mechanism of MOX for both methanol and formaldehyde.

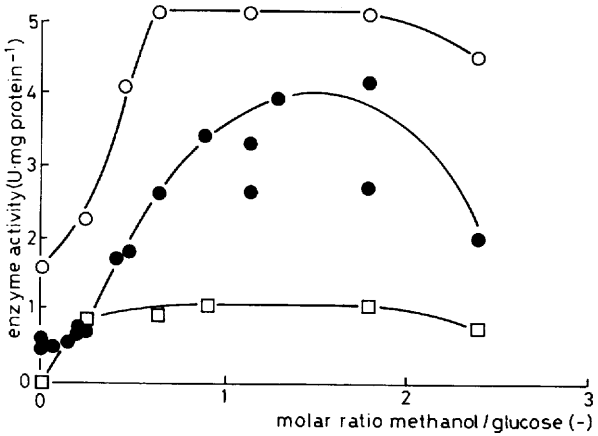

Fig. 1 Dependency of enzyme activity on molar ratio of methanol/glucose mixture of carbon-limited chemostat growing at D = 0.1 h^{-1}
MOX (●), FaDH (O) · 5, FoDH (□) · 4.

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Also FaDH and FoDH levels increased with increasing amounts of methanol in the medium feed (Fig. 1). FaDH was induced from a basal level of 0.32 U·mg protein^{-1} during growth on glucose to 1.06 U·mg protein^{-1} at a molar ratio of methanol/glucose of 0.6. FoDH increased from a very low level of 0 up to 0.24 U·mg protein^{-1}. These patterns are similar to those observed with cultures grown on formaldehyde/glucose and formate/glucose mixtures (Giuseppin et al. 1988). Formaldehyde or formate could not be detected in steady-state cultures. Only at the high molar ratio of 2.4, small amounts of methanol (5 mmol·l^{-1}) were found. This indicates that the maximal oxidation capacity for formaldehyde and formate is not the factor that limits the capacity of the cells for for methanol utilization. Experiments to adapt the catalase-negative mutant to growth on methanol as sole carbon source were unsuccessful and led to washout of the culture.

The maximal hydrogen peroxide-detoxification rate ($\underline{r}H_20_{2max}$) can be calculated by the formula: $\underline{r}H_20_{2max} = \underline{D} \cdot R/\underline{Y}sx$; R stands for the maximal molar ratio methanol/glucose; Ysx for the cell yield under those conditions expressed as g dry weight biomass per mol glucose. The values used were D = 0.1 h^{-1}, R = 2.4 (as a moderate estimate for the maximal molar ratio, Fig. 1) and Ysx = 93.6 g biomass·(mol glucose)$^{-1}$. These results show that the catalase-negative mutant detoxifies hydrogen peroxide under steady-state conditions up to at least 2.6 mmol hydrogen peroxide ·h^{-1} ·g biomass^{-1}. Under these steady-state conditions the methanol concentration was low. Since under steady-state conditions the rate of methanol oxidation, and hence the rate of hydrogen peroxide destruction, is limited by the residual substrate concentration in the culture, it was of interest to study the behaviour of cells in the presence of excess methanol. This is described in the following paragraphs.

Response of steady-state cultures grown on methanol/glucose mixtures to methanol pulses

The hydrogen peroxide-decomposition capacity of the mutant was determined with samples of continuous cultures growing at D = 0.1 h^{-1} on a methanol/glucose mixture with a molar ratio of 1. To these cultures methanol was added at various concentrations. No hydrogen peroxide

production could be detected after methanol pulses lower than 75 mM. The formaldehyde production rate was low up to a 75-mM methanol pulse, indicating the efficient conversion of formaldehyde to $C0_2$ by FaDH and FoDH. No significant levels of formic acid could be detected in these experiments (Figs. 2, 3).

Methanol pulses of 100 and 300 mM rapidly killed the culture and initiated the accumulation of hydrogen peroxide production into the medium. The viability data (Fig. 4) clearly show that the cells remain unimpaired after small methanol pulses (< 75 mM). Pulse experiments with ethanol gave similar results (data not shown). The rapid decrease in viability at high methanol concentrations could be prevented by adding a small amount of catalase prior to the pulse (Fig. 4). Apparently, exogenous catalase can keep the intracellular hydrogen peroxide concentration down at a non-toxic level. This indicates that hydrogen peroxide is the primary lethal compound rather than formaldehyde, which was also produced in substantial amounts when high concentrations of methanol were present (Fig. 3).

The above results clearly show that the excretion of hydrogen peroxide by the cells after a pulse is dependent on the initial methanol concentration. This is due to the fact that cells exhibit a poor affinity for methanol. The rate of methanol-dependent oxygen consumption (and thus the rate of intracellular hydrogen peroxide production) increased with increasing methanol concentrations up to 390 mM (Fig. 5). From a Hanes plot of these data, an affinity constant of the cells of 20 ± 5 mM was calculated. Besides the low affinity for methanol there are indications that the intracellular hydrogen peroxide production is also lowered by the inactivation of MOX during the pulse experiment. The linear hydrogen peroxide production rate after cell death (4.2 to 7.6 mmol· g biomass^{-1}·h^{-1}) indicates that MOX is inactivated as a result of the intracellular accumulation of high levels of both hydrogen peroxide and formaldehyde (Veenhuis et al. 1980).

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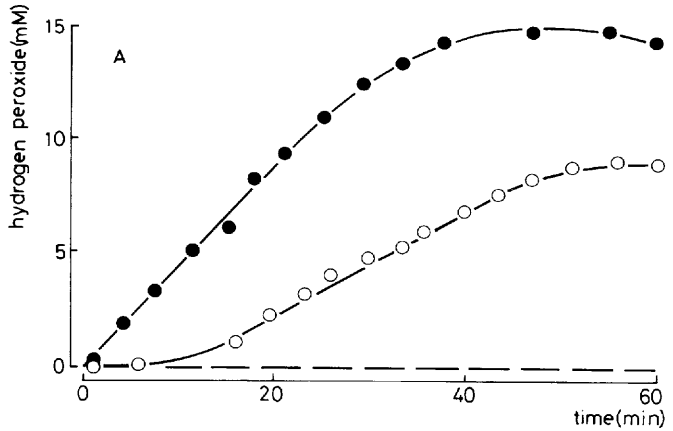

Fig. 2 Hydrogen peroxide formation by steady-state cultures grown on methanol/glucose (molar ratio 1) after injections of methanol at various concentrations. (Biomass concentration 3.3 g·l^{-1})
300-mM pulse (●), 100-mM pulse (O), 10, 30, 70-mM pulses (- - - -).

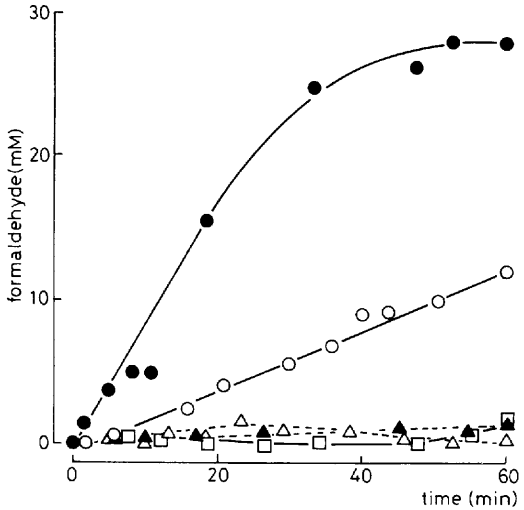

Fig. 3 Formaldehyde production by steady-state cultures grown on methanol/glucose (molar ratio 1) after methanol injections. (Biomass concentration 3.3 g·l^{-1})
300-mM pulse (●), 100-mM pulse (O), 75-mM pulse (□), 30-mM pulse (▲),10-mM pulse (△)

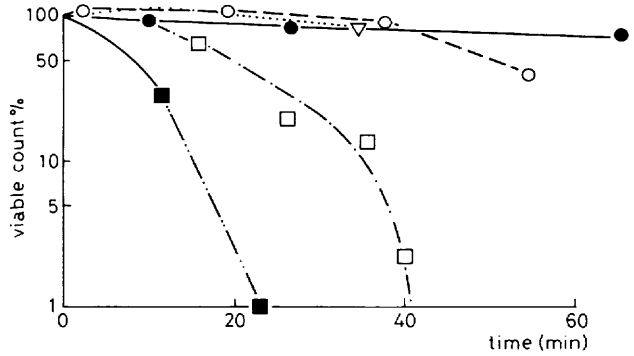

Fig.4 Viability of catalase-negative H. polymorpha after methanol injections. (Biomass concentration 3.3 $g \cdot l^{-1}$)
30-mM pulse (●), 75-mM pulse (O),100-mM pulse + catalase (▽), 100-mM pulse (□), 300-mH pulse (■).

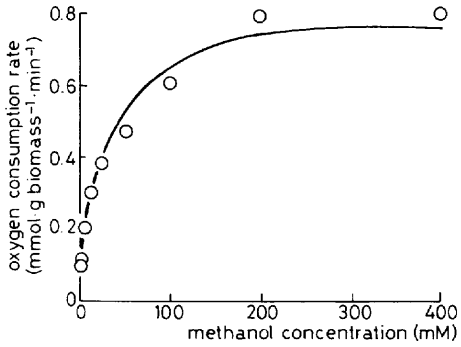

Fig. 5 Methanol-dependent oxygen consumption rate of methanol / glucose grown cells. Oxygen consumption rate was determined in a biological oxygen monitor at 37°C in an air-saturated buffer.

The sensitivity of MOX for hydrogen peroxide has been described recently for MOX ex Candida boidinii (Geissler et al. 1986). The Michaelis Menten kinetic constants for the maximal MOX inactivation rate by hydrogen peroxide (V_{max}) and the hydrogen peroxide affinity (k_a, H_2O_2) of 33 h^{-1} and 1.6 mM, respectively indicate that the stability of MOX ex Candida boidinii is poor during methanol pulses. The stability of MOX ex catalase - negative H. polymorpha to hydrogen peroxide (analysed using Michaelis Menten-type of kinetics) turned out to be higher. The V_{max} for inactivation was 1.3 ± 0.3 h^{-1}; k_a, H_2O_2 was 8-12 mM.

Since in the pulse experiments hydrogen peroxide was not excreted by the cells at, or below a concentration of 75 mM methanol, it follows from Figs. 3 and 5 that the capacity of the hydrogen peroxide destruction system is at least 3.9 mmol hydrogen peroxide · g biomass$^{-1} \cdot h^{-1}$ under transient conditions as compared to 2.6 mmol hydrogen peroxide · g biomass$^{-1} \cdot h^{-1}$ under steady-state conditions.

Effect of cultivation conditions on hydrogen peroxide production

The cultivation conditions and the state of the cells had a profound effect on the hydrogen peroxide-decomposition capacity of the catalase-negative mutant. This is evident from experiments in which cells grown on formaldehyde/glucose (molar ratio 1) were tested for methanol tolerance under conditions as described above. In contrast to the methanol / glucose-grown cells, cells grown on the formaldehyde/glucose mixture showed a hydrogen peroxide accumulation in the culture after a lag phase at a low methanol pulse of 30 mM (Fig. 6). The detoxification system can be inactivated by inactivating the cells by mild treatments such as freeze-drying. Freeze-dried formaldehyde/glucose grown cells did not show the lagtime in hydrogen peroxide production, as observed in steady-state cultures (Fig. 6).

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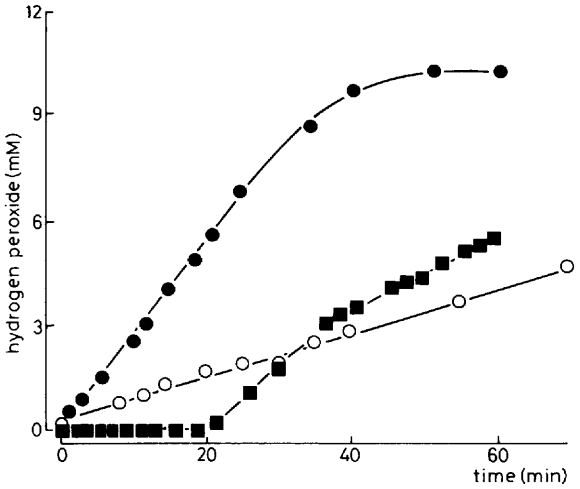

Fig. 6 Hydrogen peroxideformation by cells grown on formaldehyde/ Glucose (molar ratio 1)after methanol pulses (biomass concentration 3.3 $g \cdot l^{-1}$ for fresh cultures and 1 $g \cdot l^{-1}$ for suspension of freeze-dried cells)
100-mM pulse(●), 30-mM pulse (■),
30-mM pulse to a suspension of freeze-dried cells (O).

Discussion and conclusions

Although methanol leads to the intracellular formation of (toxic) hydrogen peroxide, the catalase-negative mutant of H. polymorpha is capable to detoxify hydrogen peroxide up to a high rate. Moreover the catalase-negative mutant can grow in continuous cultures on methanol containing media as an additional carbon source. This proves that the catalase-negative mutant of H. polymorpha must possess an alternative hydrogen peroxide-destruction system.

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The key enzyme for methanol oxidation, MOX, was induced up to relatively high levels of 40 - 50% of that of the wild-type strain. The optimal molar ratio of methanol/glucose of 1 - 2 for MOX synthesis in continuous cultures is similar to that found by other workers (Egli 1980, Egli et al. 1982). Although the wild-type and the catalasenegative strain of H. polymorpha have similar induction patterns the maximal level of MOX is lower in the catalase-negative strain. This has also been found for growth of this strain on formaldehyde/glucose and formate/glucose mixtures (Giuseppin et al. 1988). Whether this lower expression of MOX activity for the catalase-negative mutant is caused by a lower gene expression or by inactivation of MOX by intracellular hydrogen peroxide is not yet clear. The expression of the dissimilatory enzymes FaDH and FoDH is in line with that of the wild-type strain, with an expression of respectively 70 and 100% of that of the wild-type strain.

The hydrogen peroxide-detoxification rate by the mutant was remarkably high. At D = 0.1 h^{-1}, a methanol/glucose mixture with a molar ratio of 2.4, still resulted in stable steady state cultures. This corresponds with a high hydrogen peroxide-decomposing activity of at least 2.6 mmol hydrogen peroxide·g $biomass^{-1} \cdot h^{-1}$. Studies on the effects of a methanol pulse to a steady-state continuous culture showed that the maximal detoxification rate is at least 3.9 mmol hydrogen peroxide·g $biomass^{-1} \cdot h^{-1}$ (Figs. 2, 3). Methanol/glucose-grown cultures could even survive methanol pulses up to 75 mM. Addition of catalase to the culture allowed the survival at even higher concentrations of methanol. Apparently, extracellular catalase generates a gradient of hydrogen peroxide across the cell wall large enough to prevent accumulation of toxic levels of hydrogen peroxide.

The excretion of hydrogen peroxide by the cells (Fig. 3) with concurrent loss of viability (Fig. 4) are dependent on both the rate of hydrogen peroxide production by alcohol oxidase and the rate of peroxide destruction by an hitherto unidentified enzyme system. The rate of hydrogen peroxide production is dependent on the concentration of methanol and oxygen and the amount of active alcohol oxidase. From the methanol pulse experiments with cells pre-grown on a methanol/glucose mixture it was evident that only at high rates of methanol oxidation, as a result of a high methanol concentration (Fig. 5) the rate of peroxide production exceeded

that of its destruction. Remarkable differences in hydrogen peroxide production were observed in methanol pulse experiments with cells pre-grown on methanol/glucose as compared to formaldehyde/glucose-grown cells. At a methanol concentration of 100 mM, the rate of hydrogen peroxide production was similar (4.2 mmol·g biomass^{-1}·h^{-1}) for methanol/glucose and formaldehyde/glucose pre-grown cells (Figs. 2 and 6). However, at 30 mM methanol the methanol/glucose grown cells did not produce hydrogen peroxide, whereas, after a lag phase, considerable amounts of hydrogen peroxide were produced by cells pre-grown in formaldehyde/glucose. Since the amounts of MOX were approximately the same for these two types of cultures (4 to 5 U·mg protein^{-1}) it follows that the higher rate of hydrogen peroxide production by the formaldehyde/glucose-grown cells must result from a lower level of the hydrogen peroxide-destruction system. The occurence of a distinct lag phase at 30 mM methanol with formaldehyde/glucose grown cells (Fig. 6) probably results from an inactivation of the peroxide-destroying system until a critical level is reached.

The nature of the peroxide-destroying enzyme system in the catalase-negative mutant remains to be elucidated. Preliminary experiments have indicated that glutathion peroxidase (E.C. 1.11.1.9) is absent in the mutant. This enzyme may function as a peroxide destroying system in animal tissue and some fungi (Smith and Schrift 1979; Aisaka et al. 1982). However, high levels of cytochrome C peroxidase have been detected in the catalase-negative mutant of Hansenula polymorpha (Verduyn et al., to be published). It would therefore be of interest to study the level of this enzyme as a function of cultivation conditions. If cytochrome C peroxidase would be the enzyme responsible for hydrogen peroxide destruction by the mutant, thus allowing growth on methanol / glucose mixtures, it seems likely that the enzyme cannot withstand freeze-drying. Freeze-drying of cells drastically enhanced the production of hydrogen peroxide during methanol pulses (Fig. 6). The hydrogen peroxide detoxification mechanism and the induction of MOX described may have potential technical applications. It enables the production of other catalase-free (peroxisomal) oxidases, e.g. amino-oxidase and oxidases introduced by genetic engineering even if the inducing substrate leads to production of otherwise lethal hydrogen peroxide (Unilever patent 1986).

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Chapter 5

CELL WALL STRENGTH OF HANSENULA POLYMORPHA IN CONTINUOUS CULTURES IN RELATION TO THE RECOVERY OF METHANOL OXIDASE

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Summary. The changes in cell wall strength of Hansenula polymorpha have been investigated in continuous cultures with respect to the recovery of methanol oxidase (MOX). Cultures grown on several substrate mixtures that enable induction of MOX have been compared with cultures grown on methanol as the sole inducer. The effects of dilution rate (D) on lysis properties have been studied. The cell wall strength was consistently influenced by growth media and D. Media containing glycerol/methanol showed the slowest lysis kinetics, with a large fraction of non-degradable cell wall material. In continuous cultures grown on a mixture of glucose and methanol both the resistance to zymolyase and the mean cell wall thickness increased at $D < 0.1\ h^{-1}$. The yield of MOX by zymolyase lysis is reproducible and up to 100% higher than that of the standard ultrasonic treatment. The lysis kinetics indicated that zymolyase punctures the cell wall; since the release rate of MOX is lower than that of protein, the cell contents will leak through. At D-values > $0.2\ h^{-1}$, both protein and MOX release rates increase, reflecting a change in lysis mechanism due to the increased fraction of thin daughter cells. Kinetic analysis of zymolyase lysis using both physical and enzymatic methods provides information for achieving optimal recovery of MOX.

Introduction

Methanol oxidase is formed in continuous cultures of H. polymorpha grown on methanol or mixtures of methanol and another carbon source. Our optimisation study of this fermentation process is largely based on the maximal productivity principle (P/V), described by: P/V (in g MOX$\cdot h^{-1} \cdot l^{-1}$) = $D \cdot X \cdot R \cdot St \cdot C_{MOX}$, in which P=product concentration in lysate, V=volume of the continuous fermenter [1], D=dilution rate [h^{-1}], X=biomass concentration [$g \cdot l^{-1}$], R=recovery efficiency [-], St=stability of enzyme [-], and CMOX=MOX activity [MOX units$\cdot g\ X^{-1}$]. The present paper focuses mainly on the recovery efficiency (R).

The recovery of intracellular enzymes such as MOX depends largely on the cultivation method and substrates used. Many mechanical (Baratti et al. 1978) and enzymatic disruption methods are more efficient in case of weak cell walls. Less stringent methods with less inactivation of the enzyme can improve the recovery.

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The lysis of H. polymorpha is difficult (Baratti et al. 1978) and seems to be more difficult than that of other yeasts, e.g. Saccharomyces (Petersen 1985). The cell wall strength and thickness are known to be reduced at high growth rates (> 0.2 h^{-1}) (Bruinenberg 1985). At high growth rates a larger number of cells will consist of newly budded cells having a thin cell wall. At low growth rates (< 0.1 h^{-1}) the maturation of the cell wall will increase its strength because of branching of the cell wall polymers.

The composition of the cell wall depends on the nutrients used. For the methylotropic yeast Hansenula, however, only scarce data are available on the effects of dilution rate and carbon source on the cell wall strength and recovery of intracellular proteins (Baratti et al. 1978). Mathematical models describing cell wall strength have been reported for physical methods such as homogenizers and continuous-flow ultrasonic disintegrators (James et al. 1972). An overall constant for first-rate release can be calculated by these methods. The rate constant strongly depends on the apparatus used. However, an analysis of enzymatic lysis to obtain a model that describes cell wall properties has not been reported yet.

In our study of H. polymorpha in continuous cultures we describe the cell wall changes as a function of D by enzymatic lysis characteristics and measurements of cell wall thickness from electronmicrographs. In addition, we present a model to evaluate the kinetic data.

Materials and methods

Organism and growth conditions

Strain. Hansenula polymorpha CBS 4732 (normal wild-type strain).
Media. As described by Egli (1980). The carbon sources were sterilized separately by filtration. A mixture of anti-foaming agent (Rhodorsil R426, Rhone Poulenc) and concentrated ammonia in a ratio of 4 to 1 was used to control pH and to prevent foaming.

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Cultivation. H. polymorpha was cultivated in continuous cultures using a Chemoferm fermenter (Chemoferm, Sweden) with a working volume of 1.5 or 2.5 l. The cultivation conditions were 37°C, pH 5.0; the dissolved oxygen concentration was kept above 25% saturation by adjusting the air flow. Biomass samples of steady states were collected in a vial cooled to 5°C and washed twice with distilled water. The wet cell mass was stored at -32°C, which did not affect the lysis characteristics.

Lysis

Zymolyase method. 200 ml Cell suspensions with an absorbance at 610 nm (A 610 nm) of 15 to 18 corresponding with 5.5 to 6.6 g dry cells l^{-1} were made in a solution containing 0.1 M sodium phosphate pH 8.5, 5 mM EDTA, 1mM dithiotreitol and 10 mg zymolyase 100000 ex Arthrobacter luteus (Seikagaku Kogyo Co, Japan). The solution was stirred gently in a thermostatted vial at 37°C. At regular intervals of 10 or 15 min, samples were taken and analysed for MOX activity, protein content and A 610 nm. The high-purity zymolyase was used to avoid possible proteolytic activity of the enzyme preparation during cell lysis.
Ultrasonic treatments were carried out in 20-m1 Pyrex tubes using 5 ml of cell supsension in 0.1 M sodium phosphate buffer pH 7.5, cooled to 0°C in an ice bath. The cell suspension contained 0.12 g wet cells and 3 g glass beads (Sigma, 100-150 gym). The lysis was carried out with a Branson cell disruptor, type B12 (70 W) with a 3 mm microtip. The procedure consisted of up to six treatments of 1 min with cooling periods of 30 s in between the sonifications.

Assays

The MOX activity was determined according to Van Dijken et al. (1976), the activities being expressed as μmol 0_2-min^{-1}. All data have been corrected for catalase effects; one MOX unit corresponds to 16.66 nkat.

The protein level was determined according to Lowry et al. (1951), Bovine serum albumin was used as standard.

The biomass level was determined by dry weight measurements (drying at 110°C for 16 h) and by measuring the A 610 run with a Vitatron colorimeter in 1-cm cuvettes in an appropriate dilution.

Cell structure. Electronmicrographs were made of deepfrozen samples embedded in EPON and stained for active peroxisomes with $CeCl_3$ (Veenhuis et al. 1976). The dimensions of the cell structures were estimated from electronmicrographs by taking the mean values of ten to twenty cells per steady state.

Data analysis. The data were fitted to the equations with the standard nonlinear curve-fitting technique by means of the Marquard optimisation routines.

Results and discussions

Comparison of lysis methods

The ultrasonic and the zymolyase procedure have been compared by measuring the steady-state MOX activity of the cells after lysis (Fig. 1). The zymolyase method was further investigated by measuring the protein release rate and A 610 run during lysis (Fig. 2), and the MOX release rate during lysis at several dilution rates (Fig. 3). The reproducibility of the ultrasonic treatment was not very high. The use of specific MOX activity expressed as activity per mg protein might compensate for the less efficient cell break-up. The zymolyase treatment tends to be more efficient that the ultrasonic treatment.

Modelling of the zymolyase mediated cell lysis

Protein release and optical density. Quantitative evaluation of the lysis data requires a model that describes the lysis kinetics sufficiently. Under the experimental conditions - with excess of zymolyase - we use quasi-first-order kinetics. The zymolyase lysis

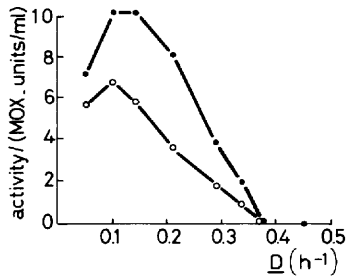

Fig. 1. The effect of lysis method on MOX activity. Substrate: glucose / methanol 4:1 w/w,
● zymolyase method O ultrasonic method

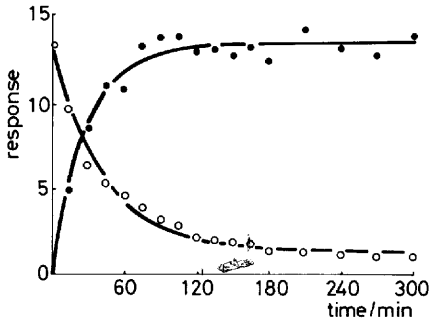

Fig. 2. Absorbance at 610 nm (A 610 nm) and protein release rate (kp) during lysis of H. polymorpha by zymolyase.
Substrate glucose/methanol 4:1(w/ w).
O A 610 nm ● protein $g.l^{-1}\cdot 5$

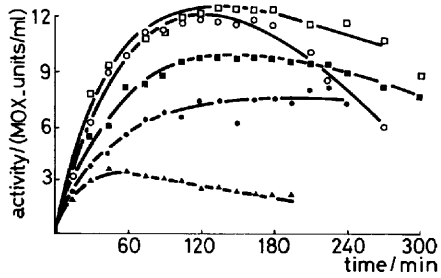

Fig. 3. MOX release rate during lysis of H. polymorpha by zymolyase at various dilution rates. Substrate glucose /methanol 4:1 (w/w). D-values (h^{-1}):
● 0.05 □ 0.1 O 0.14
■ 0.21 ▲ 0.29

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reaction with respect to protein release can be described by the firstorder reaction [protein](t) = [$protein_{max}$]·[1-exp(-k_p·t)] in which k_p is the release rate constant of protein (min^{-1}); the decrease of A 610 nm can be described by:
A 610 nm (t) = A 610 nm_{max}·exp(-k_c·t) + A 610 nm residual, in which kc is the release rate constant of A 610 run (min^{-1}).
The above formulas gave a good fit for all experimental data of protein release and cell decay. A typical example is given in Fig. 2. The low mean values of the normalised standard deviation for the curves studied (0.4 A610 nm units and 0.14 $g \cdot l^{-1}$ protein) indicate the adequacy of the model.

MOX release and activity. The MOX release was modelled by another equation as some samples required compensation for the decay of MOX activity at long incubation times due to proteolytic activity of the lysate and the possible presence of minor contaminants in the zymolyase preparation. To minimize the latter activity, we used a highly purified zymolyase preparation. However, no differences in MOX stability were found when using zymolyase preparations of either 6000 U/mg or 10000 U/mg.
Analysis of the MOX release curves revealed that the first-order decay of MOX occurred after a considerable incubation time. We assume that MOX initially comes out of the cell in aggregates (as undamaged peroxisomes), which are not very sensitive to proteolytic attack; then these aggregates dissociate into protease-sensitive MOX:

intracellular →	free, stable →	unstable →	inactive
MOX	MOX	MOX	MOX

In their simplest form the three sequential differential equations are:

$$\frac{d[\text{intracellular MOX}]}{dt} = -k_{MOX} \cdot [\text{intracellular MOX}] \qquad \text{[Eq. 1]}$$

$$\frac{d[\text{stable MOX}]}{dt} = k_{MOX} \cdot [\text{intracellular MOX}] - k_{dis} \cdot [\text{stable MOX}] \qquad \text{[Eq. 2]}$$

$$\frac{d[\text{unstable MOX}]}{dt} = k_{dis} \cdot [\text{intracellular MOX}] - k_{d} \cdot [\text{unstable MOX}] \qquad \text{[Eq. 3]}$$

in which k_{MOX}=release rate constant of MOX activity [min^{-1}], k_{dis} = dissociation rate constant [min^{-1}] and k_d=decay constant of MOX activity [min^{-1}].

The MOX activity measured in time equals the sum of the activities of [stable MOX] and [unstable MOX). The analytical solution of [Eqs. 13] can be written as:

$$MOX(t) = A \cdot \exp(-k_{MOX} \cdot t) + B \cdot \exp(-k_d \cdot t) + C \cdot \exp(-k_{dis} \cdot t) \qquad \text{[Eq. 4]}$$

in which

$$A = \frac{MOX_{max} \cdot k_{MOX}}{k_{dis} - k_{MOX}} \cdot \left(1 + \frac{k_{dis}}{k_d - k_{MOX}}\right) \qquad \text{[Eq. 4a]}$$

$$B = \frac{MOX_{max} \cdot k_{dis} \cdot k_{MOX}}{(k_{MOX} - k_d) \cdot (k_{dis} - k_d)} \cdot \qquad \text{[Eq. 4b]}$$

$$C = \frac{-MOX_{max} \cdot k_{MOX}}{k_{dis} - k_{MOX}} \cdot \left(1 + \frac{k_{dis}}{k_d - k_{dis}}\right) \qquad \text{[Eq. 4c]}$$

In [Eq. 4] we assume the third term containing C to be constant (C*) compared to the second term containing k_d ($k_d < k_{dis}$). The following equation, having a first-order decay rate, gave a satisfactory fit for all data analysed:

$$MOX(t) = A \cdot \exp(-k_{MOX} \cdot t) + B \cdot \exp(-k_d \cdot t) + C^* \qquad \text{[Eq. 5]}$$

Effect of D on recovery

Zymolyase susceptibility, The determination of MOX activity by monitoring a zymolyase-treated cell suspension during lysis gives information on the cell wall strength under different culture conditions.

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Zymolyase was used in concentrations that were not rate-limiting. Some data on MOX activity of H. polymorpha are given in Fig. 1. More data will be published by Giuseppin et al. (1988).

Fig. 4 - in which the first-order rate constants have been plotted - shows how much the zymolyase susceptibility depends on the growth rate. At low D values the fraction of the residual cell density, compared to the initial cell density, indicates a maturation effect of the cell wall caused by the large fraction of nondegradable cell walls. The residual A 610 nm is caused by large cell membrane fragments and not by undamaged cells, as judged by examination using a light microscope. Similar results for zymolyase susceptibility have been found for Candida utilis by Bruinenberg (1985).

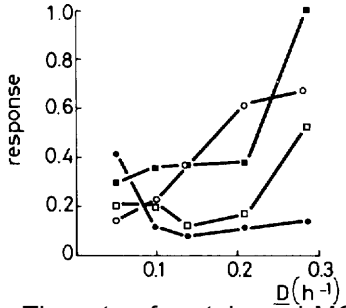

Fig.4. Kinetics of the lysis by zymolyase of H. polymorpha. Substrate: glucose/methanol 4:1 w/w.
O k_c min^{-1}-10 ● A 610 nm/A 610 nm at t=0 □ k_{MOX} min^{-1}·10 ■ k_p min^{-1}·10

The rate of protein and MOX release is constant up to D=0.2 h^{-1}. At D>0.29 h^{-1}, however, the release rates increase significantly. On the other hand the cell lysis rate increased monotonically by a factor of six. The protein release rate is higher than those of cell lysis and MOX release. These differences between the rates of release and decay indicate either the release of some periplasmatic protein, or a leakage of protein from partly damaged cells, which is fast compared to the release of peroxisomes from the cells. At D-values > 0.2 h^{-1}, the contribution of young and very sensitive cells can be clearly seen by the strong increase of the protein release rate.

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Decay of MOX activity. The sensitivity of the lysate to proteolytic or other forms of breakdown increases significantly at D-values > 0.1 h^{-1} as can be seen from the significant change of kd in Eq. 5 (the inactivation term). As is shown by Fig. S, the calculated values of kd increased from $6 \cdot 10^{-5}$ min^{-1} at D=0.1 h^{-1} to $3 \cdot 10^{-4}$ min^{-1} at D=0.29 h-l.

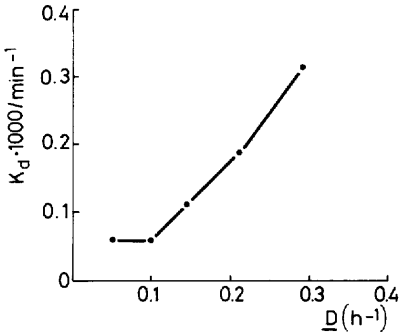

Fig. 5. MOX inactivation rate as a function of dilution rate during lysis by zymolyase of H. polymorpha. Substrate glucose/methanol 4:1 (w/w).

Cell dimensions

Cell wall thickness was studied using electronmicrographs of samples at different D-values. Fig, 6 illustrates the difference in cell wall thickness between the mother and daughter cell at D=0.1 h^{-1}. At higher D-values the difference between mother and daughter cell decreases. The mean cell wall thickness decreases from 0.2 to 0.12 pm if D increases from 0.05 to about 0.24 h^{-1}. It can be seen that, apart from zymolyase susceptibility, both cell wall thickness and maturation of the cell are of great importance for the cell wall strength at low D-values.

To determine the effect of peroxisome size on MOX activity in the lysate, estimates have been made by measuring the mean peroxisome dimensions using electronmicrographs. The size of $CeCl_3$-positive peroxisomes slightly decreased from about 0.8 μm at D=0.1 h^{-1} to 0.5 μm

at D=0.35 h^{-1}. At D-values > 0.35 h^{-1}, only the $CeCl_3$-positive spots that were nonperoxisomal could be detected. This nonperoxisomal activity accounts for 1-2% of the residual MOX activity detected in "in vivo" assays. The "in vivo" activity is difficult, to isolate quantitatively in the cell lysate, both with the zymolyase assay and ultrasonic treatment as used by e.g. Egli (1980). Lysis by passage through a French press does not inactivate this nonperoxisomal MOX activity (Eggeling and Sahm 1978).

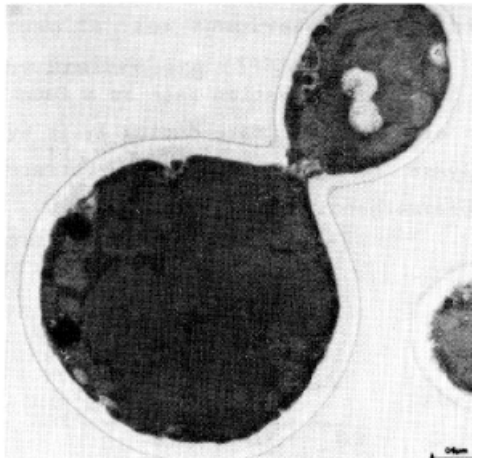

Fig. 6. Difference in cell wall thickness of mother and daughter cell.

Effect of carbon sources

The cell wall susceptibility depends strongly on the carbon source used. Cells grown in mixtures of glycerol or glucose and methanol tend to have thicker walls compared to methanol-grown cells (Table 1).

Table 1. Susceptibility to zymolyase of H. polymorpha grown on different carbon sources[a]

Carbon source	k_c [min^{-1}]	k_{MOX} [min^{-1}]	k_p [min^{-1}]	A 610 nm of residual fraction[b]	mean cell wall thickness [μm][c]
glucose/methanol 4:1	0.022	0.02	0.036	0.11	0.17
methanol	0.0057	0.0050	0.0084	0.31	0.13
glycerol/methanol 3:1	0.0017	0.0029	0.0027	0.44	0.19

a D=0.1 h^{-1}
b A610 nm / A610 nm at t=0
c standard deviation = ± 0.03

Conclusions

The effects of growth rate on cell wall strength do not solely depend on cell wall thickness. The cell wall strength is probably more influenced by the degree of cross-linking during the maturation of the cell wall rather than by other effects of growth rate. The carbon source used strongly influences the susceptibility to zymolyase. It is remarkable that the cell wall resistance of methanol-grown cells is larger than that of glucose / methanol-grown cells.

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The zymolyase method to lyse H. polymorpha is a good alternative to the ultrasonic treatment since it yields a higher MOX activity. The cost of zymolyase is likely to be a limiting factor in large-scale isolations; this problem, however, can be solved by combining the zymolyase method with the ultrasonic method or other physical treatments. With respect to the cell break-up step in downstream processing, glucose/methanol mixtures are the most satisfactory carbon sources in MOX production.
In the overall MOX production process, the knowledge of the cell strength properties may lead to the selection of suitable process conditions to improve the recovery of the intracellular enzyme.

Abbreviations and symbols

C_{MOX}	MOX activity	[MOX units·g X^{-1}]
D	dilution rate	[h^{-1}]
MOX	methanol oxidase	
k_c	decay rate constant of A 610 nm	[min^{-1})
k_d	decay constant of MIX activity	[min^{-1}]
k_{dis}	dissociation rate constant	[min^{-1}]
k_{MOX}	release rate constant of MOX activity	[min^{-1}]
k_p	release rate constant of protein	[min^{-1}]
R	recovery efficiency of enzyme	[-]
St	stability of enzyme	[-]

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Chapter 6

MATHEMATICAL MODELLING OF GROWTH AND ALCOHOL OXIDASE PRODUCTION BY HANSENULA POLYMORPHA GROWN ON METHANOL/GLUCOSE MIXTURES

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SUMMARY

Growth and production of methanol oxidase (MOX) by Hansenula polymorpha (CBS 4732) has been modelled to provide a mathematical description of such production processes. Two kinds of mathematical models were constructed, one for growth on methanol and one for growth on mixtures of methanol and glucose. The model for growth on methanol as the sole carbon source consists of kinetics expressions for a limited number of key steps concerning substrate and product inhibition. This model was used to predict and simulate the culture dynamics at the start-up, the most critical step in continuous cultivation. The growth on mixtures of methanol and glucose was modelled assuming virtually independent metabolic pathways for the two compounds. The induction and production of MOX could be described by adaptation of various induction and repression equations for a number of data from the literature. These models for growth and MOX production describe both experimental data and literature data on growth of H. polymorpha (CBS 4732) on glucose / methanol mixtures satisfactorily. All parameters for the induction / repression model for growth of H. polymorpha CBS 4732 on glucose / methanol mixtures yielded evidence that a similar induction / repression pattern is involved in MOX production. Catalase, however, is repressed by a different mechanism.

INTRODUCTION

The regulation of alcohol oxidase (MOX) synthesis and the physiology of methylotrophic yeasts has been studied extensively for the last ten years by using continuous cultivation techniques.
The regulation of the expression of the MOX gene is highly complex and until now for H. polymorpha three compounds with MOX-inducing capacities have been found: methanol, formaldehyde and formate[1]. Ethanol, glucose and a number of other sugars have proven to be potent repressors. In cultures of H. polymorpha grown on mixtures of glucose and methanol the repression by glucose can be partly overcome[2].

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MOX, which is located in organelles called peroxisomes, mediates the conversion of methanol into H_20_2 and formaldehyde. H_20_2 is decomposed by catalase, which is also located in the peroxisomes. The formaldehyde is either assimilated via the xylulose monophosphate route[2,3] or dissimilated to $C0_2$, yielding two molecules of NADH per molecule formaldehyde.

A complication in large scale cultivation of methylotrophic yeasts is their sensitivity to disturbances caused by accumulating inhibitory levels of methanol, formaldehyde and formic acid[4,5].

In this study we aimed to develop and subsequently evaluate a model describing the growth and culture dynamics of Hansenula polymorpha CBS 4732 and also of MOX production by this organism cultivated in batch and continuous cultures with either methanol or mixtures of glucose and methanol as carbon and energy sources.

THEORY

Dynamics of cultures grown on methanol

MOX in cultures of H. polymorpha CBS 4732 grown on methanol as the sole carbon source is almost fully induced over a wide range of dilution rates[6]. The dynamics of the culture growing on methanol are determined by the maximum consumption rates of methanol, formaldehyde and formic acid.

The maximum methanol consumption rate is about 0.42 g methanol·g biomass^{-1}·h^{-1} [7,8], The maximum formaldehyde consumption rate in continuous cultures of H. polymorpha DL1 after a pulse of formaldehyde is 0.66 g formaldehyde·g biomass^{-1}·h^{-1} [9]. The non-dissociated form of formic acid proved to be the most toxic intermediate that occurs either during or after a process disturbance at pH 4 to 4.7[9]. The value of the inhibition constant, $\underline{k}_{i,Fo}$, is about 0.18 g l^{-1} [9]. At pH values of 4.8 to 6.3, above the pKa of formic acid, this value is higher[9]: Pilat and Prokop[4] found a value of approx. 3 g·l^{-1} for Candida boidinii 11 Bh at pH 5.0 in batch cultures. However, the growth characteristics of the strain H. polymorpha CBS 4732, differ from those of H. polymorpha DL1 and Candida boidinii 11 Bh.

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Many aspects of methanol metabolism have been quantified on the level of the individual enzymes involved[3]. However, it is not preferable to implement all the reactions in one mathematical model, as this would hinder both parameter estimation and the practical use of the model. We will therefore restrict ourselves to a description of growth by reactions related directly to the MOX reactions with the key compound formaldehyde at the branching point of dissimilation and assimilation. This scheme can be summarized as:

$$\begin{array}{ccccccc} & & \text{rMOX} & & \text{rFaDH} & & \text{rFoDH} \\ \text{methanol} & \rightarrow & \text{formaldehyde} & \rightarrow & \text{formate} & \rightarrow & \text{C02} \\ & & \downarrow\ \underline{\text{r}}\text{a} & & & & \\ & & \text{biomass} & & & & \end{array}$$

The growth of H. polymorpha on methanol can be described by combination of the substrate and product-inhibited growth rate equations:

$$\mu = \frac{\mu_{max,Me} \cdot [Me]}{\underline{k}_{a,Me} + [Me] + [Me]^2/\underline{k}_{i,Me}} \cdot \frac{\underline{k}_{i,Fa}}{\underline{k}_{i,Fa} + [Fa]} \cdot \frac{\underline{k}_{i,Fo}}{\underline{k}_{i,Fo} + [Fo]} \qquad (1a)$$

Recently Luong reported a modified kinetic equation for n-butanolinhibited growth of Candida utilis[10] and the above model can be rewritten using the linear form proposed by Luong[10].

$$\mu = \frac{\mu_{max,Me} \cdot [Me]}{\underline{k}_{a,Me} + [Me]} \cdot \left[1 + \frac{[Me]}{\underline{k}_{i,Me}}\right] \cdot \left[1 + \frac{[Fa]}{\underline{k}_{i,Fa}}\right] \cdot \left[1 + \frac{[Fo]}{\underline{k}_{i,Fo}}\right] \qquad (1b)$$

The differential equations describing the biomass formation and the methanol consumption in continuous cultures are as follows:

$$\frac{d[x]}{dt} = \underline{r}_x - \underline{D} \cdot [x] \qquad (2)$$

$$\frac{d[Me]}{dt} = -\underline{r}_{MOX} + \underline{D} \cdot ([Me0] - [Me]) \qquad (3)$$

$$\frac{d[Fa]}{dt} = r_{MOX} - r_a - r_d - D \cdot [Fa] \quad (4)$$

$$\frac{d[Fo]}{dt} = -r_d - r_{FoDH} - D \cdot [Fo] \quad (5)$$

with:

$r_{MOX} = r_{MOXmax} \cdot [Me] \cdot [x] \cdot (k_{MOX} + [Me])^{-1}$
$r_{FoDH} = r_{FoDHmax} \cdot [Fo] \cdot [x] \cdot (k_{a,Fo} + [Fo])^{-1}$
$r_a = \Phi \cdot \mu \cdot [x] \cdot y_{Me,x}^{-1}$
$r_d = r_{FaDHmax} \cdot [Fa] \cdot [x] \cdot (k_{aFa} + [Fa])^{-1}$
$r_x = \mu \cdot [x]$

In steady states the following rates are related with a constant fraction of formaldehyde that is assimilated, Φ :

$r_d = (1 - \Phi) \cdot r_{MOX}$, and $r_a = \Phi \cdot r_{MOX}$

and, assuming that the Pirt-equation can be applied, the equation

$$r_a + r_d = r_x \cdot (y_{Me,x})^{-1} + m_{Me} \cdot [x] \quad (6)$$

Eqs. 1-6 can be used to determine the dynamics of continuous cultures in phase planes analysis. In some experiments it is assumed that $r_{FoDHmax}$ and r_{MOXmax} (MOX, FaDH and FoDH are induced) are larger than r_a, and that the growth rate is determined by the methanol consumption rate only.

Modelling of growth on glucose/methanol mixtures

Models for the growth of microorganisms on mixed substrates have been described in the literature using both structured and unstructured models[11,12]. The models may include mechanisms in which the metabolisms of the two substrates are optimally controlled with respect to growth rate[11]. The degree of interaction of the metabolism of two substrates can vary to a large extent, e.g. Bader[12]. The growth of H. polymorpha in continuous cultures on mixtures of glucose and methanol is characterised by a nearly independent uptake and metabolism of the two substrates over a wide range of glucose/methanol ratios and dilution rates [13-15].

To construct the model, the following assumptions were made:

a. the growth of H. polymorpha on glucose can be described by ideal Monod kinetics[13,16];
b. the growth of H. polymorpha on methanol can be described by substrate-inhibited growth kinetics (as in eqs. 1a or 1b) if the methanol flux in the cell is higher than an arbitrarily chosen value of 99% of the total carbon flux. In cases of lower methanol flux, ideal Monod kinetics can be assumed. The latter can be deduced from data by Egli[17] and Giuseppin et al.[8], who found no decrease of the maximum specific growth rate when growing H. polymorpha on glucose/methanol mixtures in the presence of high concentrations of residual methanol (up to 5 $g \cdot l^{-1}$);
c. the yield coefficients for growth on glucose and methanol are constant, and each coefficient is independent of the other substrate. They can be used according to ratio of the two carbon fluxesl[18];
d. the glucose uptake (r_G) follows Monod kinetics:

$$r_G = \frac{r_{Gmax} \cdot [G]}{[G] + k_{a,G}} \quad (7)$$

e. the methanol uptake rate (rMe) in a steady or quasi-steady state culture can be described by either Monod or substrate-inhibited uptake kinetics as under b.;
f. the biomass formation can be described by:

$$r_x = \mu \cdot [x] \quad (8)$$

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Data by Egli et al.[14] and Eglil[7] indicate that the growth rate can be described by the independent contributions of the glucose and methanol fluxes. The maintenance substrate consumption can be divided into two contributions proportional to the glucose/methanol ratio.

$$\mu = -y_{G,x} \cdot - y_{Me,x} \cdot r_{Me} - mG \cdot R - mMe \cdot (1-R) \qquad (9)$$

μ. is limited by the maximal rate of metabolism, which is equal to the growth rate on glucose.

This upperlimit for μ can be deduced from the closely related pathways involved in the metabolism of methanol and glucose. Although the first reactions of their assimilation differ, they share the same metabolism at the level of dihydroxy acetone phosphate. The rate of assimilation beyond the level of dihydroxy acetone phosphate will determine the μ max and will be less or equal to the growth rate on glucose.

$$\mu \leq \mu_{max,G} \text{ (on glucose)} \qquad (10)$$

Assuming an independent metabolism we can calculate steady state conditions using modified eqs. 3 to 6, yielding two sources of biomass: xG for glucose, and xMe for methanol. Summarizing:
[x] = [xG] + [xMe]. The steady-state substrate concentrations can be calculated using the terms xG and xMe.

Data by Eggeling and Sahmlg and Egli et al.[14] have shown that, if the culture is grown on methanol/sugar mixtures at high growth rates, a constant fraction of methanol (0.61) is dissimilated. This also occurs at growth rates higher than those for growth on methanol alone. This indicates that the xMe compartment grows faster and more efficient at higher glucose/methanol ratios. During growth on methanol (R = 0), the maximal methanol consumption rate is 0.42 -0.44 g Me·g x^{-1} · h^{-1}. At increasing glucose/methanol ratios, the closely linked metabolic pathways of glucose and methanol result in a more efficient xMe compartment with an increased specific methanol consumption rate. The xMe compartment size itself decreases as the glucose level rises.

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The increase of the methanol consumption rate in the xMe compartment is assumed to be linear, the level similar to that of the glucose consumption rate being the upper limit.

$$rMe,max - KMe,max^{*} \text{ ' } (1-R) + R \cdot rG,max \qquad (11)$$

* maximal specific methanol consumption rate in methanol-grown cultures (g Me · g x^{-1} · h^{-1})

In non-steady states, the rMe,max is limited by the rate of methanol oxidation by MOX in the cell, rMOX, or:

$$r_{Me,max} \leq rMOX \qquad (11a)$$

The methanol consumption rate also depends on the degree of induction (Q) for the biomass and is discussed subsequently. Therefore eq. 11 should be multiplied by Q. The MOX activity in the cell at low Dvalues is to a great extent in excess of the methanol consumption rate [6,8]. The effect of Q has initially been neglected in the models.

Modelling of induction repression in MOX production

Many methods have been described to model induction and repression phenomena in microbial culturesl[9]. Most of these models assume a direct correlation between an inducer or an energy potential in the cell and the observed induction or repression. The regulation of MOX induction is very complex. In building a simple and suitable model for process description, the induction and formation of MOX are assumed to be determined by two nearly independent, genetically tuned processes in terms of induction and repression.

In case MOX is induced in the cells, methanol is the most potent inducer. The intermediates formaldehyde and formic acid can also be regarded as inducers[1,15]. On other carbon sources, (catabolite)

repression may occur. Ethanol is the strongest repressor, followed by glucose, sorbitol and glycerol[20].

In modelling, the effective concentration of inducer or repressor is supposed to determine the MOX formation. The actual effect of methanol in the cell is enhanced by the MOX-inducing metabolites formaldehyde and formic acid. The induction/repression hypothesis can be modelled using several equations from the literature[19,21,22]. It has been reported, that during growth in continuous cultures on glucose/methanol mixtures, high levels of MOX-mRNA are formed up to high dilution rates (< 0.3 h^{-1}), although the formation of MOX-protein decreased dramatically at increasing dilution rates[8]. Furthermore, the MOX-activity appeared to be very unstable at increasing dilution rates[25], which may be caused by increased proteolytic activity in the cell.

These data suggest two independent, counteracting, processes in the cell. Induction of two enzym systems are involved, one of which lead to induction of MOX and one of which leads to inactivation of MOX. This may seem highly inefficient for the cell. However, these cultivation conditions (steady state growth on glucose/methanol at high growth rates) are rare in nature. This may be seen as an inadequate response of the yeast under these artificial conditions.

For modelling purposes these two processes have been used. A simple dual control system as described by Todal9 for other repression and induction phenomena in continuous cultures can not be used because the system involves induction of two genetically independent processes.

The model of Yagil and Yagil[21] is used since it allows description of two independent, genetically tuned processes:

- the induction of MOX by methanol. This leads to MOX-mRNA in the presence of the repressor glucose[8];
- the induction of MOX-directed proteolytic acitivity at high glucose concentrations[24,25].

The induction of the proteolytic system is assumed to be the main cause of MOX inactivation. This process will be called repression.

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The basic equations are derived from a relative enzyme induction (Q), which is defined as (d[MOX] / d[x]) / $\{d[MOX] / d[x])_{max}$. The repression can be described by the equation:

$$Q1 = \frac{\underline{k}_d \cdot Q_{b1} \cdot [G]^{hr} + Q_{b1}}{1 + Q_{b1} + \underline{k}_d \cdot Q_{b1} \cdot [G]^{hr}} \qquad (12)$$

The induction can be described by the equation:

$$Q2 = \frac{Qb2/kd2 \cdot [Me]^{ha} + Q_{b2}}{1 + Q_{b2} + Q_{b2}/\underline{k}_{d2} \cdot [Me]^{ha}} \qquad (13)$$

The overall expression, Q, can be calculated by:

$$Q = Q1 \bullet Q2 \qquad (14)$$

Evaluation of the applicability of the above equations requires measurement of the concentration of effective methanol. However, measurements of very low methanol concentrations in the culture will not give the correct concentration for effective methanol in the cell, since the metabolites of methanol, formed by the conversion of methanol, act as MOX inducers as well.

EXPERIMENTAL

Organism and growth conditions

Strain. Hansenula polymorpha CBS 4732.
Medium. As developed by Egli[17] except that the antifoaming agent PPG was omitted from the medium. The medium was sterilized at 120°C for 20 min. Vitamins and methanol were sterilized separately by filter sterilization. The inlet concentration of the carbon source was 10 $g \cdot l^{-1}$ unless stated otherwise.

Cultivation. H. polymorpha was cultivated in continuous cultures using a Chemoferm fermenter (Chemoferm, Sweden) with a working volume of 1.5 to 2.5 l. The temperature was maintained at 37 ± 0.2°C; and the pH was kept at pH 5.0 ± 0.05 by addition of an ammonia solution (35% w/w) containing the antifoam Rhodorsil R426 in a ratio of 4:1 (v/v). The medium feed was introduced together with the air supply using one nozzle to improve the mixing of the substrate in the culture, which was especially necessary at low D-values. The oxygen tension was kept above 25% saturation. Steady states were determined by measuring the respiration parameters and the MOX activity levels in whole-cell suspensions and in the cell lysate.

Assays

The MOX activity in cell lysates was determined according to Giuseppin et al.[8]. The oxygen consumption rate in 5 ml potassium phosphate buffer 0.1 M, pH 7.5, containing 0.1 ml sample, and 40 mM methanol was measured at 37°C. The activity was calculated as micromole methanol consumed per minute. "In vivo" activity of washed cell suspension was measured as described above except that 80 mM methanol was used.
Glucose and methanol were determined enzymatically[26]. Methanol concentrations < 10 $mg \cdot kg^{-1}$ were determined by GLC.
Formaldehyde was determined by the colorimetric method of Lang and Lang27.
Dry weight levels were determined after drying a washed cell suspension at 110°C for 16 h. Estimations of biomass in biochemical assays were made by determining the optical density (OD) at 610 nm in 1-cm-cuvettes; the values were converted into dry weight using a calibration curve.
The protein level was determined according to Lowry et al.[28]. Bovine serum albumin was used as standard.

Numerical simulations

Numerical simulations were performed according to the the ISIS simulation software package (Simulation Sciences), using a Sarafyan six-stage fifth-order routine with a variable step. Model estimates were calculated using programs written in FORTRAN 77 with NAG library subroutines.

DEVELOPMENT AND EVALUATION OF THE MODEL

Kinetic model for growth on methanol as the sole carbon source

Start-up of the continuous culture. The parameters of the equations describing the growth dynamics of H. polymorpha CBS 4732 on methanol were determined in batch experiments. The constants $k_{i,Me}$ and $k_{a,Me}$ were determined in batch cultures using inocula adapted to methanol. The initial growth rates, given in Fig. 1, fitted very well to eqs. 1a and 1b. The effects of Fa and Fo have not been incorporated as only the initial growth rates were determined. In both cases μ_{max} was 0.21 h^{-1}. The $k_{a,Me}$-values were 0.12 and 0.105 $g \cdot l^{-1}$ for eqs. 1a and 1b respectively. These values are identical to the values reported for H. polymorpha DL-129. The $k_{i,Me}$ values of 17.5 and 34.6 $g \cdot l^{-1}$ yield a growth rate of half the wax at a methanol concentration of 17.5 and 17.3 $g \cdot l^{-1}$ for eqs. Ia and Ib respectively. This indicates a higher methanol inhibition effect compared with H. polymorpha DL-1, which showed half the μmax at 29 $g \cdot l^{-1}$ methanol[5].

Model (equation) 1b describes the data slightly better than does model 1a. However, the difference in residual sum of squares ($1.2 \cdot 10\text{-}3$ for 1b and $1.86 \cdot 10^{-3}$ for 1a) is not statistically significant (N=21). Under practical conditions, i.e. methanol concentrations lower than 5-10 $g \cdot l^{-1}$, the two models describe the kinetics equally well. At high residual methanol concentrations equation 1b is preferred.
The equations can be used to calculate the biomass-substrate (xs) phase planes for various conditions of the continuous culture. The first bottleneck for an efficient continuous cultivation of H. polymorpha grown on methanol is the time the process takes to reach *a* steady state. The above models were used to study the feasibility of a straightforward

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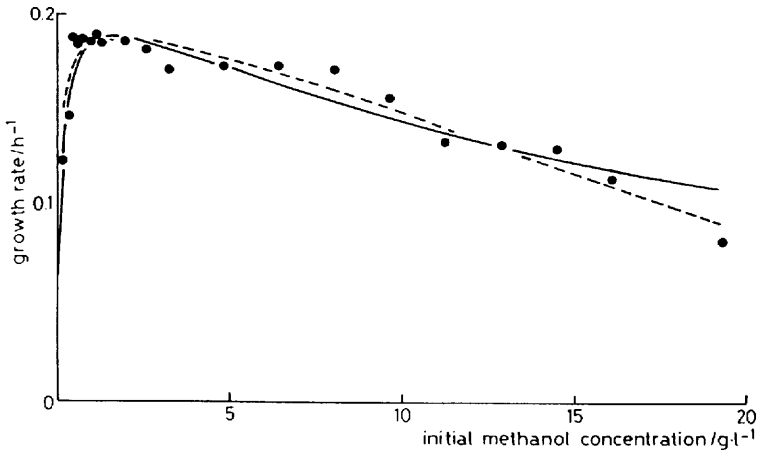

Fig. 1. Growth rate of H. polymorpha on methanol in batch cultures at various methanol concentrations; predicted vs. experimental data. ● = experimental; ____- =model 1; - - - - = model 1b

start-up procedure. Several start-up procedures were tested with a view to obtaining a rapid start-up for continuous cultures with high cell densities (30-60 $g \cdot l^{-1}$) without computer control.

Starting arbitrarily with batch cultures up to 4 $g \cdot l^{-1}$ biomass, the medium feed pump was set at a D-value in the range of 0.01 to 0.1 h^{-1}. The highest D-value leading to a steady state was 0.045 h^{-1}. This value is close to the maximum start-up D-value as calculated by the numerical simulations (Fig. 2). Maximal D-values of 0.046 and 0.045 were calculated using eq. 1a and eq. 1b, respectively. From the simulations of the start-up procedure (Fig. 2a and b) it can be seen that the contribution of the formaldehyde and formate accumulation term is rather small. The parameters used for the simulations were either determined experimentally or estimated from data from the literature on various enzymatic activities (Table 1). Fig. 2c shows the accumulation of formaldehyde and formate during the batch phase. The patterns are

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similar to those for Candida boidinii 11 Bh4. The parameters used for the simulation of growth on methanol are given in Table 1.

Eqs. 1-6, though without induction kinetics, can well be used since the induction values for MOX, FaDH and FoDH in the batch phase are already high before start-up.

Studies on the dynamics of methanol-grown cultures. The growth dynamics of H. polymorpha in continuous cultures have been described by Swartz and Cooney5. We studied in detail the inhibition by methanol and formaldehyde under our cultivation conditions (pH 5).

Experiments using pulse methanol additions were carried out to determine the recovery rate of the culture. To steady states of continuous cultures growing at D = 0.1 h^{-1}, methanol was added to a final concentration of 0.2-0.9 $g \cdot l^{-1}$. The methanol and formaldehyde conversion rate can be described by the following simplified first-order reactions.

$$d[Me]/dt = -D \cdot [Me] - k_{Me} \cdot [x] \qquad (15a)$$

$$d[Fa]/dt = -D \cdot [Fa] - k_{Fa} \cdot [x] \qquad (15b)$$

A typical trace of the experimental data for the methanol and the formaldehyde concentration after a pulse of methanol is given in Fig. 3. A plot of the logarithm of the concentrations in time results in a straight line. Equation 15a, b fit the data well down to 4 and 0.2 mg. l^{-1} for respectively methanol and formaldehyde. At lower concentrations the linear relations can no longer be applied.

The first-order reaction rates at [x] = 14 $g \cdot l^{-1}$ were 1.6 g Me· $h^{-1} \cdot g\ X^{-1}$ and 0.59 (g $Fa \cdot h^{-1} \cdot g\ X^{-1}$ for methanol (kMe) and formaldehyde (k_{Fa}) respectively. The formaldehyde uptake rate is close to the value of 0.66 g $Fa \cdot h^{-1} \cdot g\ X^{-1}$ as found by Swartz[5]. The in vivo MOX activity at about 100 air saturation gives a maximal methanol uptake rate of 3.7 g $Me \cdot h^{-1} \cdot g\ X^{-1}$. The k_{Me} determined in continuous cultures (1.6 g $Me \cdot h^{-1} \cdot g\ X^{-1}$ at

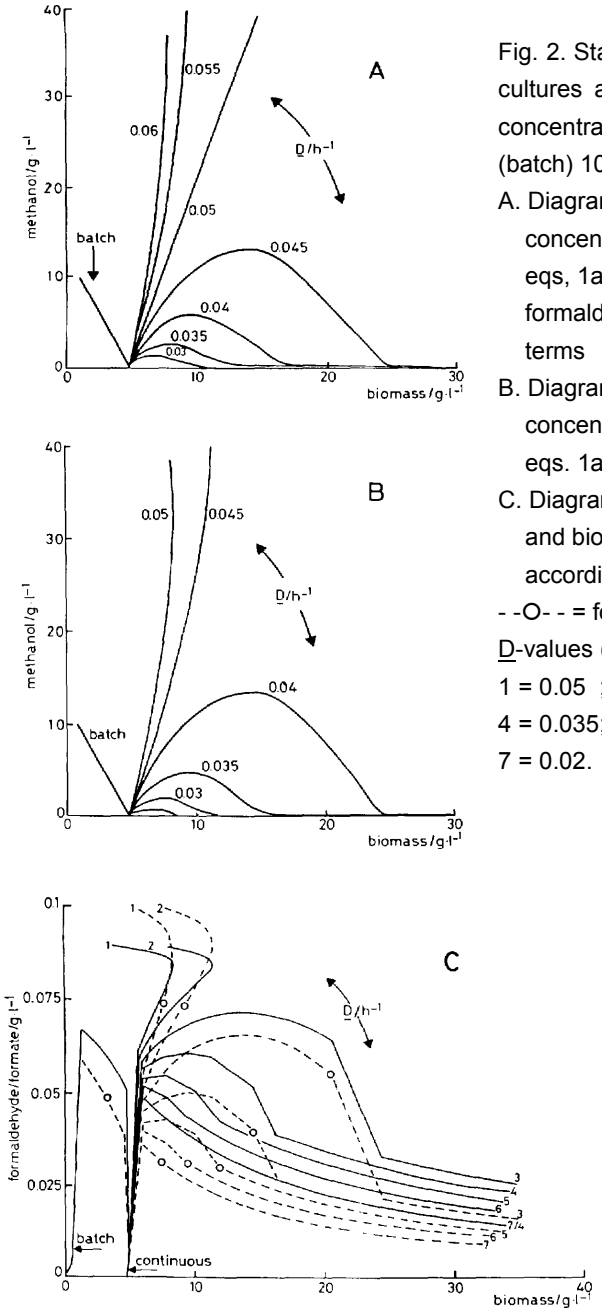

Fig. 2. Start-up simulation of continuous cultures at various dilution rates. Start concentration: biomass $g \cdot l^{-1}$; methanol (batch) 10 $g \cdot l^{-1}$ methanol (feed) 100 $g \cdot l^{-1}$

A. Diagram of methanol and biomass; concentration according to the model: eqs, 1a and 2-6 without formaldehyde and formate inhibition terms

B. Diagram of methanol and biomass concentration according to the model: eqs. 1a and 2-6

C. Diagram of formate/formaldehyde and biomass concentration according to model eqs. 1a + 2-6

- -O- - = formate; __ = formaldehyde.

D-values (h^{-1}):

1 = 0.05 ; 2 = 0.045; 3 = 0.04 ;
4 = 0.035; 5 = 0.03 ; 6 = 0.025;
7 = 0.02.

Table 1, Parameters used for the simulation of growth of H. polymorpha on methanol; data either determined experimentally or obtained from the literature

parameter	value/ unit	Source
$k_{a,Fa}$	0.075 $g \cdot l^{-1}$	Schutte et al.[30]
$k_{a\ Fo}$	0.60 $g \cdot l^{-1}$	Schutte et al.[30]
$k_{a,Me}$	0.12 $g \cdot l^{-1}$	Eglil[7]
$k_{a,\ MOX}$	0.03 $g \cdot l^{-1}$	van Dijken et al.[6]
$K_{i\ Fa}$	0.5 $g \cdot l^{-1}$	Pilat and Prokop[4], Swartz and Cooney[5]
$k_{i,Fo}$	3.0 $g \cdot l^{-1}$	Pilat and Prokop[4], Swartz and Cooney[5], Swartz[9]
$k_{i\ Me}$	17.5 $g \cdot l^{-1}$	this study
mMe	0.012 g Me·g $x^{-1} \cdot h^{-1}$	Giuseppin et al.[8]
$r_{FaDHmax}$	0.66 g Fa·h^{-1}·g x^{-1}	this study
$r_{FoDHmax}$	0.5 g Fo·h^{-1}·g x^{-1}	Pilat and Prokop[3]
r_{MOXmax}	0.44 g Me·h^{-1}·g x^{-1}	Egli et al.[7]
	0.39 (-)	Eggeling and Sahm[18]
μmax	0.21 h^{-1}	this study

30% air saturation) is close to the value of 1.4 g Me·h^{-1}·g X^{-1} calculated after correction for the effect of oxygen on MOX activity, using a pseudo-Michaelis-Menten equation with an affinity constant for oxygen of about 0.4 $mmol \cdot l^{-1}$.

Estimation of biomass yields for growth on mixtures of methanol and glucose

Theoretical and experimental data on growth and MOX production of H. polymorpha on a glucose/methanol mixture of 4:1 were compared using

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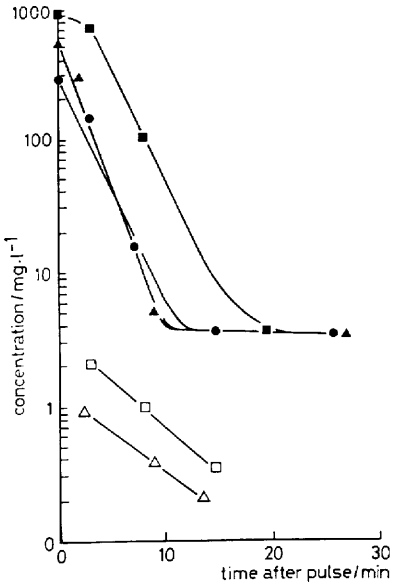

Fig. 3 Methanol and formaldehyde traces during pulse additions of methanol to a steady state continuous culture
D = 0.1 h^{-1}, [x] = 14 $g \cdot l^{-1}$.
● = methanol pulse, initial conc. 286 mg $\cdot l^{-1}$;
▲ = methanol pulse, initial conc. 537 $mg \cdot l^{-1}$;
△ = formaldehyde formed after ▲;
■ = methanol pulse, initial conc. 860 $mg \cdot l^{-1}$;
□ = formaldehyde formed after ■

eqs. 7-14. The substrate concentrations were calculated with the parameters
$k_{a,G}$ = 0.015 $g \cdot l^{-1}$, μ_{maxG} = 0.52 h^{-1}, y_{Gxmax} = 0.52 g x·g G^{-1},
$k_{a,Me}$ = 0.12 $g \cdot l^{-1}$, $\mu_{max,Me}$ = 0.21 h^{-1}, y_{MOXmax} = 0.42 g x·g Me^{-1},
$k_{i,Me}$ = 17.5 $g \cdot l^{-1}$.

The theoretical residual methanol concentration, based on the above mentioned parameters, as a function of D at several glucose/methanol ratios is given in Fig. 4. The profile of the calculated methanol concentrations is similar to that of comparable experiments with Kloeckera[2]. The absolute concentrations calculated are higher than those reported; this may be due to methanol absorption to the cells or high affinity for methanol by MOX in the cell. The $k_{a,MOX}$ for methanol

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is 0.03 $g \cdot l^{-1}$ compared to the $k_{a,Me}$ for growth of 0.12 $g \cdot l^{-1}$. The reported $k_{a,s}$ values for H. polymorpha are higher than those for the Kloeckera strain[2]. The actual $\mu_{max,Me}$ on glucose/ methanol mixtures were calculated by varying the Ymax,Me according to eq. 11.

The biomass formation was calculated by assuming an independent substrate consumption and utilisation rate, as given in eqs. 7-14. The equations, having the above parameter values, were verified by data reported by Egli et al.[7]. These workers presented a compilation of steady-state biomass yields and methanol and glucose concentrations. The maximal biomass yield on glucose, $y_{Gx,max}$, of 0.54 g x·g G^{-1}, is slightly higher than the value found in our study (0.52). The model fits well as can be seen in Fig. 5 a,b. The main discrepancies occur at low glucose / methanol ratios at high D-values. The residual methanol concentration, under those conditions, results in a slightly decreased biomass formation, but not in a decreased maximum growth rate[16].

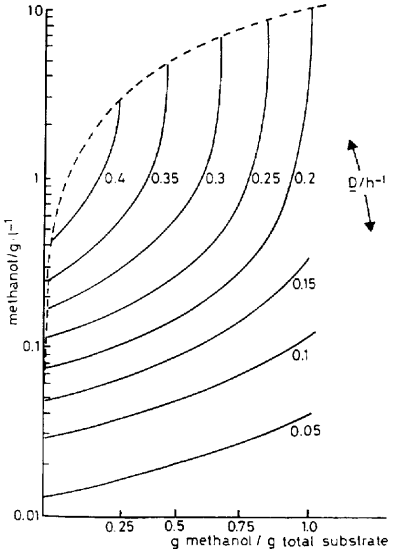

Fig. 4. Residual methanol concentrations for H. polymorpha cultures grown on glucose/methanol. $k_{a,G}$ = 0.015 $g \cdot l^{-1}$;
$k_{a,Me}$ = 0.12 $g \cdot l^{-1}$. μ_{Me} = 0.21 h^{-1},
μ_G = 0.52 h^{-1}; - - - = wash-out.

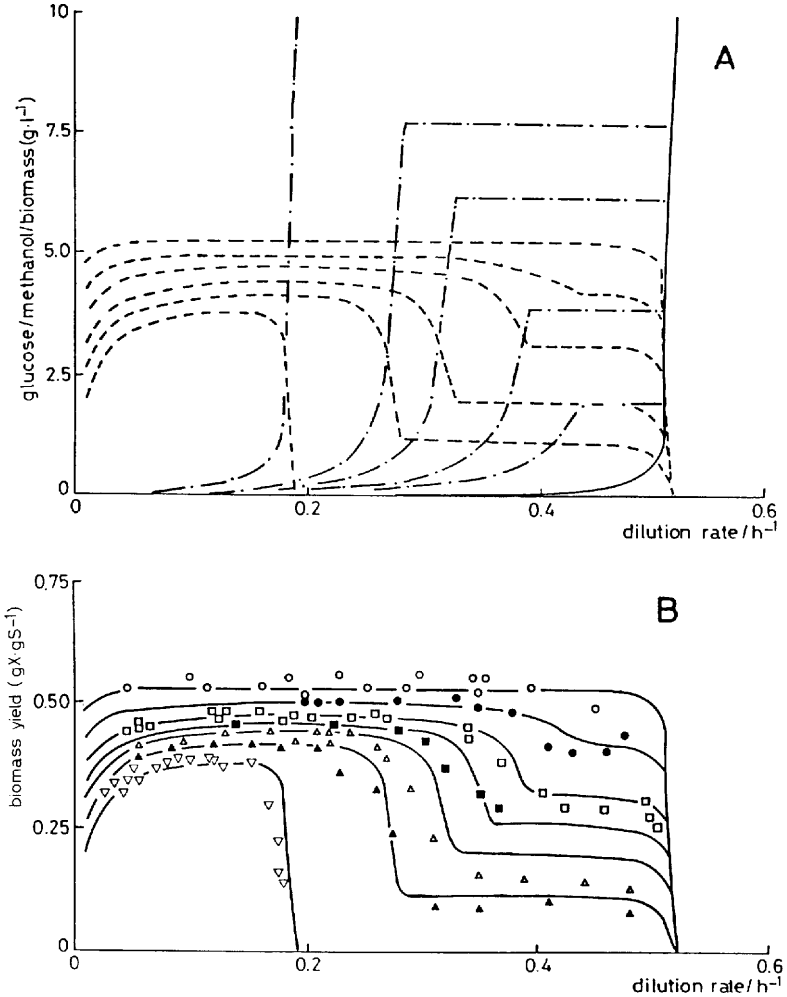

Fig. 5. A. Simulation of biomass, residual methanol and glucose concentrations (parameters obtained from Egli et al.[14]); ___ = glucose; -●-●= methanol; - - - - = biomass

B. Fitting of models 7-14 to data by Egli et al.[7,14]

O = 0 % methanol;·
● = 19.3% methanol;
□ = 39.0% methanol;
■ = 49.5% methanol;
▲ = 77.4% methanol;
△ = 61.8% methanol;
▽ =100 % methanol.

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Egli et al.[14] found a decrease of $y_{G,x,max}$ from 0.54 down to 0.48 g x·g G^{-1} when the residual methanol concentration was increased up to 5 g·l^{-1}. However, this decrease of biomass yield due to methanol was not observed in continuous cultures of the metylotrophic yeast Kloeckera sp. 2201[16].

Induction/renression model for growth on methanol and glucose

Quantification of the model. The models for growth and MOX regulation in cultures grown on mixtures of glucose/methanol, i.e. eqs. 12-14, were quantified using data from previous experiments with continuous cultures of H. polymorpha[8] grown on a glucose/methanol mixture (4:1, w/w) (Fig. 6). The values of the parameters were determined by plotting ln s against ln {(Q/(1-Q)-Q_b}, and by linear regression according to Yagil and Yagil[21] (Table 2). The residual concentration was calculated as described in the previous section. The curves generated by the model have been plotted in Fig. 7. The model gives a good description of the experimental data at both low and high dilution rates.

The model has also been tested for other data for the same strain of Hansenula polymorpha CBS 4732. Using data from other sources[3,13,16-18], and applying similar methods, parameter values comparable to those of this study were found (Table 2, Fig. 8). Fig. 8 shows that the lines for glucose/methanol and sorbitol/methanol are close together. There are, of course, several assumed model parameter values, e.g. the affinity constants k_{Me} and k_G. The term kdl depends on the estimate of k_{Me} or k_G. It can be corrected for other estimates of the affinity constants by kd, corrected = k_{d1} table · k_s new/k_s, table. The exponent of the Q-functions are independent of the ks estimates, as the ks values do not change the slope of the plots. Surprisingly the exponent of the repression term (nr for these data) is -3.05 ± 0.14 (Table 2), which is within the error for all sources, even when different substrate ratios are used. The glucose repression is caused by three equilibria with an operon. In general, four of these equilibria are found for catabolite

Table 2. Parameters of induction/repression model for various substrate mixtures; data either determined experimentally or obtained from the literature ($\underline{k}_{a,G}$ = 0.015 g·l^{-1}, $\underline{k}_{a\ Me}$ = 0.12 g·l^{-1})

substrate mixture	source	nr (-)	na (-)	kd1 (g·l^{-1})	Q_b (-)	$Q_b \ln(Q_b \cdot \underline{k}_{d1})$ (-)
MOX formation						
glucose/ methanol 4:1	this study,	-	3.68	$3.2 \cdot 10^{-8}$	0.09	14.85*
	Giuseppin et al.[8]	-3.1	-	$5.08 \cdot 10^{-5}$	0.02	-13.8
glucose/ methanol 3.88:6.12	Egli [17]	-3.2		- $3.38 \cdot 10^{-5}$	0.01	-14.9
glucose	Egli et al.[16]	-3.04		- $9.6 \cdot 10^{-3}$	0.015	-11.15
sorbitol/ methanol 1:0.8	Eggeling and Sahm[18]	-2.92		- $2.87 \cdot 10^{-5}$	0.012	-14.88
catalase formation						
sorbitol/ methanol 1:0.%	Eggeling and Sahm[18]	-1.59	-	0.33	0.01	-5.72

*$\ln(Q_{b1}/\underline{k}_{d2})$

repression[19]. The induction of MOX, determined by the estimates of the residual methanol concentration, showed an induction term (na) of 3.68. Although this estimate is not very accurate, it can be seen that at least three to four equilibria are involved in MOX induction. The three equilibria of the operon with methanol and its direct metabolites formaldehyde and formic acid account for the induction term found.

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This model can also be applied to repression data for growth on glucose as the sole carbon source. The repression data on glucose as the sole carbon source16 show a similar na-value (approx. 3), but the kdlvalue, compared with those on mixtures of glucose/methanol, is significantly higher. This indicates a lower affinity of the repressor/effector for the operon than that found with mixtures of methanol and glucose. Considering these values, it is likely that the repression in glucose-grown cultures follows a mechanism similar to that of the repression in glucose/methanol mixtures.

The repression of MOX found at higher D-values, i.e. high residual glucose concentrations, can also be caused by an increased proteolytic activity induced by glucose[24]. This type of catabolite inactivation was demonstrated to occur in glucose/methanol grown-cells in continuous cultures by gelelectrophoresis of cell lysates of H. polymorpha, and by analysis of MOX stability[8, 25]. The induction of a specific protease rather than a repression at the MOX-gene level may be the main contribution to the constants nr and $\underline{k}_{d1}$[8].

The parameters calculated for sorbitol/methanol and glucose/ methanol are of the same order of magnitude. (2.9 and 3.1 respectively for nr, the kdl values are approx. 2-5·10-5 g·l^{-1}), thus demonstrating that the repression of MOX can be described by the same processes. The model parameters for the repression of catalase in sorbitol/methanol grown cultures[18] differ from the MOX formation parameters. This implies that a repression mechanism other than the repression of MOX formation is involved.

The model can be used to describe literature data consistently. Alternatively, the model may also be used to predict MOX levels under certain cultivation conditions. This may be useful to simulate process conditions for optimization studies. Eqs. 7-11 were used to predict the data on MOX as presented by Egli[17]. The reasonable good fit of the predicted MOX levels to the levels observed (with respect to maximal activity) is shown in Fig. 9.

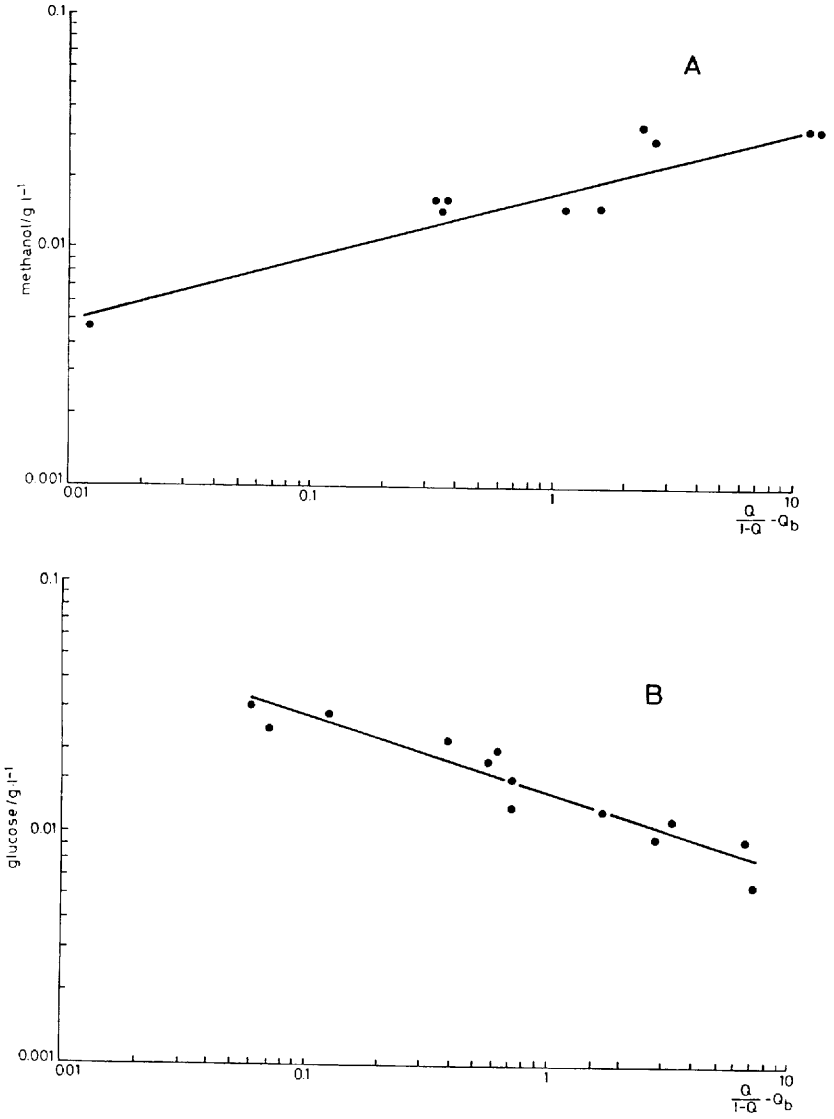

Fig. 6. A. Induction of MOX by methanol in glucose/methanol 4:1 (w/w);
B. Repression of MOX by glucose in glucose/methanol 4:1 (w/w).

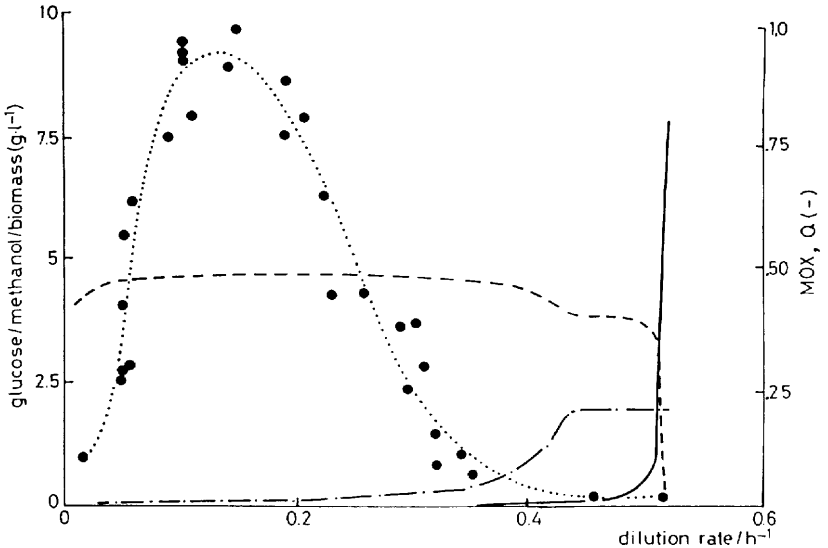

Fig. 7. Fitting of induction/repression model to data from Giuseppin et al[8].
Mixture: methanol/glucose 4:1 (w/w) . . . . . = model, - - - = biomass, - ● - ● - ● - = methanol, _____ =glucose.

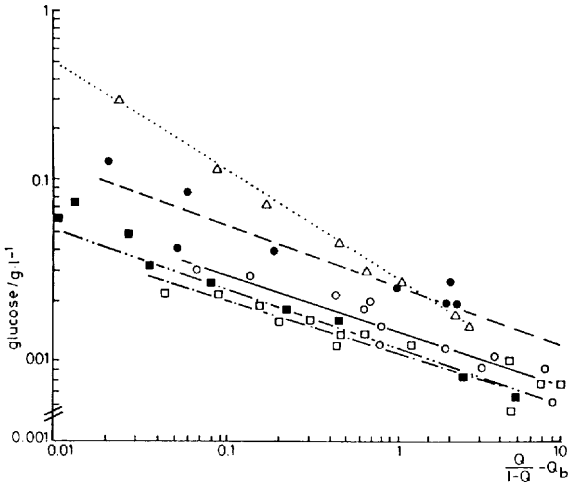

Fig. 8. Repression models: fitting of predicted values to experimental data and data from the literature.

MOX expression:

- **Glucose / methanol 4:1** (w/w),
correlation coefficient 0.94
_____ model; experimental data[8] (this study);
- **Glucose**, correlation coefficient 0.94, - - - - model, Egli et al.[13]
- **Glucose/methanol 3.88: 6.12** (w/w), correlation coefficient 0.92 - · - · - model, Egli et al.[17]
- **Sorbitol/methanol**, correlation coefficient 0.9; - ·· - model, Eggeling et al.[18]
Catalase repression: .... models, Eggeling et al.[18]

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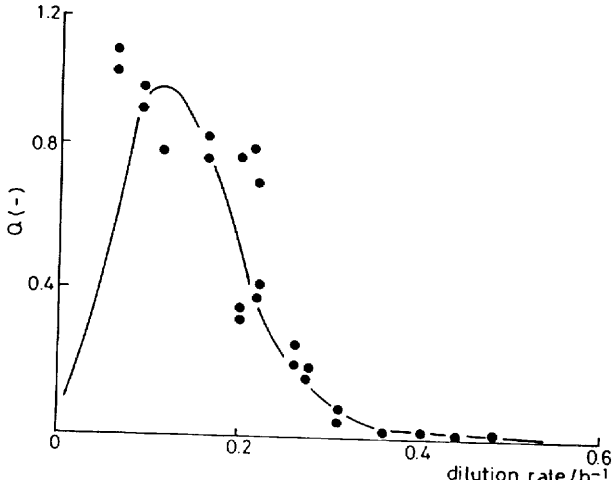

Fig. 9. Q-function: fitting of predicted values to data from Egli et al.[17]. Mixture: glucose/methanol 3.88:6.12.

Implications of the model to the fermentation process. The model (eqs. 7-14) can be used to estimate the induction level of MOX at various ratios of glucose/methanol. Using the parameters previously described (Table 2), the model is capable of globally predicting large changes in the degree of MOX e.g. induction (Q) at a D-value of 0.1 h^{-1}. See Fig. 10. This can be used in process optimization studies or controlled expression systems. For optimization studies it is also important to have an initial working model that describes the dynamics of both growth and MOX production. For production purposes, the slow kinetics of MOX formation from start-up up to productive levels can account for a nonefficient phase during the start-up of a continuous culture. A tentative dynamic behaviour of H. polymorpha cultures can be derived using the steady-state relations of eq. 12-14. The MOX production can be estimated both by means of substrate consumption rates and by synthesis of MOX in the xMe compartment in the culture. The dynamics of MOX in the culture can be described by:

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$$\frac{dMOX}{dt} = \mu \cdot Q \cdot [x] \cdot \left[\frac{[MOX]}{[x]}\right]_{max} - D \cdot [MOX] \qquad (16)$$

The dynamics of both continuous and batch cultures may be described by using the models eqs. 1-6, 12-16 as first estimates.

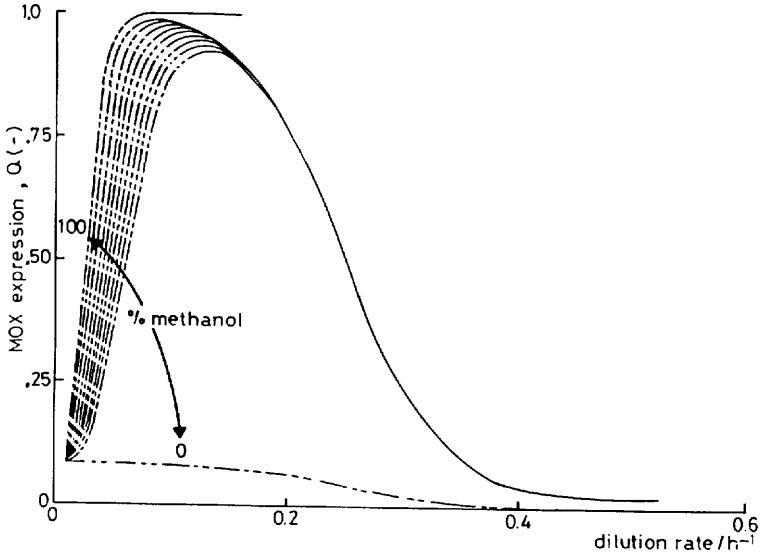

Fig. 10. Modelling of MOX induction in continuous cultures at various dilution rates and methanol/glucose ratios ranging from 0-100 % methanol.

CONCLUSIONS

Growth of and MOX production by H. polymorpha CBS 4732 can be described satisfactorily by the models proposed in this paper. They can be used to:

- give a coherent evaluation of data from the literature on several types of H. polymorpha. The model evaluation is consistent with the assumption that induction can be ascribed to methanol, and repression to glucose-induced proteolytic activity. A similar repression mechanism is found in sorbitol/methanol and glucose/methanol-grown cultures. Catalase synthesis is repressed by a different mechanism;
- predict the culture dynamics. The model can predict critical phases in the fermentation process, e.g. response to disturbances and start-up effects;
- design an effecient MOX production process. Levels of MOX expression at different dilution rates and various mixtures of glucose / methanol can be estimated;
- extend the model to enable the control or design of a (fed-)batch process for the production of MOX with H. polymorpha on various substrates.

For the development of a new enzyme production process, a good initial process model is very valuable. Though the models presented here are satisfactory, more experimental data are needed to improve them, and to extend them to other process conditions.

ACKNOWLEDGEMENT

I thank Ir. Henk Noorman for his constructive critical comments.

ABBREVIATIONS AND NOMENCLATURE

D	dilution rate	$[h^{-1}]$
Fa	formaldehyde	
FaDH	formaldehyde dehydrogenase	
Fo	formic acid	
FoDH	formate dehydrogenase	
G	glucose	
$k_{a,s}$	affinity constant (s = Me,G,Fa or Fo)	$[g \cdot l^{-1}]$
k_d	dissociation constant of repressor and operon	$[g \cdot l^{-1}]$
k_{Fa}	rate constant of formaldehyde uptake	$[g \cdot l^{-1}]$
k_{Fo}	rate constant of formate uptake	$[g \cdot l^{-1}]$
k_{MOX}	affinity constant of methanol uptake	$[g \cdot l^{-1}]$
$k_{i,s}$	inhibition constant (s = Me,Fa or Fo)	$[g \cdot l^{-1}]$
k_{Me}	rate constant of methanol uptake	$[g\ Me^{-1} \cdot g\ x^{-1}]$
Me	methanol	
mG	maintenance glucose consumption	$[g\ G \cdot g\ x^{-1} \cdot h^{-1}]$
Me0	methanol concentration at inlet	$[g \cdot l^{-1}]$
mMe	maintenance methanol consumption	$[g\ Me \cdot g\ x^{-1} \cdot h^{-1}]$
MOX	methanol oxidase	
na	number of equilibria involved in induction	
nr	number of equilibria involved in repression	
Q	relative degree of enzyme induction	
Q_b	basal expression ($=k_d/R_t$; Yagil and Yagil [21])	
R	glucose fraction in glucose/methanol mixture	$[g \cdot g^{-1}]$
r_a	assimilation rate, DHAS-determined route	$[g\ Fa \cdot h^{-1} \cdot g\ x^{-1}]$
r_d	dissimilation rate	$[g\ Fa \cdot h^{-1} \cdot g\ x^{-1}]$
r_{FaDH}	rate of formaldehyde conversion	$[g\ Fa \cdot h^{-1} \cdot g\ x^{-1}]$
r_{FoDH}	rate of formate conversion	$[g\ Fo \cdot h^{-1} \cdot g\ x^{-1}]$
r_{Me}	rate of methanol consumption	$[g\ Me \cdot h^{-1} \cdot g\ x^{-1}]$
r_{MOX}	rate of in vivo methanol consumption by MOX	$[g\ Me \cdot h^{-1} \cdot g\ x^{-1}]$
r_x	rate of biomass formation	$[g\ x \cdot h^{-1} \cdot l^{-1}]$
S	substrate	
t	time	[h]
x	biomass	
ysx	yield coefficient biomass on substrate (s = Me or G)	$[g\ x \cdot g\ s^{-1}]$
μ	growth rate	$[h^{-1}]$
Φ	relative flux	[-]

All concentrations, e.g. [Me], are expressed as: $[g \cdot l^{-1}]$

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Chapter 7

PATENT APPLICATIONS ON THE PRODUCTION AND USE OF CATALASE-FREE METHANOL OXIDASE

The data presented in chapter three and four formed the basis of two patent applications. In order to give an idea of the contents of these patent applications the abstracts and claims are given. Apart form application of MOX in detergents, these patent applications have been written in view of the potential application of catalase-free MOX and catalase-free oxidases in general.

The use of formaldehyde/glucose and formate glucose mixtures

Abstract of patent application number PCT/EP87/00295, NL8601454 priority date 05/06/86, publication number W087/07639.

The invention provides the preparation of an oxidase or oxidasecontaining mixture by aerobic fermentation of a methylotrophic yeast, wherein a catalase-negative yeast is allowed to grow in anutritive medium in the presence of a compound which induces expression of the oxidase gene but is not a substrate for the oxidase.

For example, methanol oxidase is formed by induction with formaldehyde or formiate. Examples of yeasts are Hansenula polymorpha and Pichia pastoris. Specific ratios of inducing agent and carbon source, e.g. glucose, are given. The yeasts are not capable of producing catalase, so that the oxidase produced can be used for producing hydrogen peroxide, e. g. when an alkanol is combined with methanol oxidase in a washing or bleaching process. The oxidase can also be used for qualitative and/or quantitative determination of a substrate for the oxidase, e.g. ethanol or amines, or be used as catalyst for the oxidation of a substrate in chemical synthesis.

Claims

1. Process for the preparation of an oxidase or an oxidase-containing mixture or preparation by aerobic fermentation of a yeast under conditions in which the yeast produces oxidase, and, if desired, isolation from the yeast cells of the oxidase produced,

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characterized in that a catalase-negative mutant of a yeast is allowed to grow in a nutritive medium suitable for the yeast in the presence of at least one compound which induces the expression of the oxidase gene but is not a substrate for the oxidase.

2, Process according to claim 1, in which a catalase-negative mutant of a yeast of the genus Hansenula or of the genus Pichia is used, in particular of a yeast of the species Hansenula polymorpha or of the species Pichia pastoris, more in particular the catalase-negative mutant Hansenula polymorpha 55/11, ATCC 46059.

3. Process according to claim 1 or 2, in which a yeast is used that is modified with the aid of recombinant DNA techniques, or its progenity, in which the genetic information for the oxidase stands under control of a methanol oxidase (MOX) promotor, in particular the MOX-promotor of Hansenula polymorpha CBS 4732.

4. Process according to any of the claims 1-3, in which methanol oxidase is produced as oxidase, in particular a methanol oxidase that has the same amino acid sequence as the known methanol oxidase of Hansenula polymorpha CBS 4732, or a derivative of this sequnce obtained via enzyme engineering.

5. Process according to any one of the claims 1-4, in which formiate or formaldehyde is used as the compound which induces expression of the oxidase gene but is not a substrate for the oxidase.

6. Process according to any one of the claims 1-5, in which the yeastis cultured in a continuous fermentation.

7. Process according to any one of the claims 1-6, in which the yeast is cultured in the presence of a carbon source suitable for the chosen yeast and of a formiate, the molar ratio formiate: carbon source being 0.5:1 to 4.5:1, preferably being 2:1 to 3.5:1.

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8. Process according to any one of the claims 1-6, in which the yeast is cultured in the presence of a carbon source suitable for the chosen yeast and of formaldehyde, the molar ratio formaldehyde: carbon source being 0.1:1 to 3:1, preferably being 0.5:1 to 2.5:1.

9. Process according to any one of the claims 1-8, in which the yeast is cultured in a nutritive medium that contains glucose as carbon source.

10. Process according to any one of the claims 1-9, in which the oxidase produced is isolated from the yeast cells under oxygen-free conditions.

11. Oxidase-containing, catalase-negative mutant of a yeast, obtained by a process according to any one of the claims 1-9.

12. Oxidase, obtained by a process according to claim 10.

13. Use of an oxidase-containing, catalase-negative mutant according to claim 11 or of an oxidase according to claim 12, together with a substrate for the oxidase, for the production in situ of hydrogen peroxide in a washing or bleaching process, preferably with ethanol as the substrate.

14, Washing or bleaching composition containing an oxidase-containing, catalase-negative mutant according to claim 11 or an oxidase according to claim 12.

15. Use of an oxidase-containing, catalase-negative mutant according to claim 11 or of an oxidase according to claim 12 for the qualitative and/or quantitative determination of a substrate for the oxidase.

16. Use of an oxidase-containing, catalase-negative mutant according to claim 11 or of an oxidase according to claim 12 as catalyst for the oxidation of a substrate of the oxidase within the framework of a chemical synthesis or of a process for purifying refuse.

The use of methanol/glucose mixtures.

Abstract of patent application number EP87201055, NL8602978, priority date 24/11/1986, publication number EP242007.
The invention provides the preparation of an oxidase or oxidasecontaining mixture by aerobic fermentation of a methylotrophic yeast, wherein a catalase-negative yeast is allowed to grow in a nutritive medium in the presence of:
(a) a so-called inducing substrate that induces expression of the oxidase gene and may also be substrate for the oxidase, and
(b) another source of carbon that is suitable for the yeast species chosen, the molar ratio of the inducing substrate to other source of carbon being such that the yeast formed and the oxidase formed do not suffer any harmful effects from the oxidation of the inducing substrate.

For example, methanol oxidase is formed by induction with methanol in the presence of glucose. Examples of yeasts are Hansenula polymorpha and Pichia pastoris. Specific ratios of inducing substrate and glucose as other carbon source are (0.025-3):1 and preferably (1-1.8):1. The yeasts are not capable of producing catalase, so that the oxidase produced can be used for producing hydrogen peroxide, e.g. when an alkanol is combined with methanol oxidase in a washing of bleaching process. The oxidase can also be used for qualitative and/or quantitative determination of a substrate for the oxidase, e.g. ethanol or amines, or be used as a catalyst for the oxidation of a substrate in chemical synthesis.

Claims

1. Process for the preparation of an oxidase or an oxidase-containing composition by aerobic fermentation of a yeast under conditions in which the yeast produces oxidase, and, if desired, isolation from the yeast cells of the oxidase produced, wherein a catalase-negative mutant of a yeast is allowed to grow in a nutritive medium suitable for the yeast in the presence of
(aj a so-called inducing substrate that induces expression of the oxidase gene and may also be substrate for the oxidase, and
(b) another source of carbon that is suitable for the yeast species chosen, the molar ratio of the inducing substrate to other source of carbon being such that the yeast formed and the oxidase formed do not suffer any harmful effects from the oxidation of the inducing substrate

2. Process according to claim 1, in which a catalase-negative mutant of a yeast of the genus Hansenula, preferably of the species Hansenula polymorpha, particularly Hansenula polymorpha 55/11, ATCC 46059, or of the genus Pichia, preferably of the species Pichia pastoris is used.

3. Process according to one or more of the preceding claims, in whicH. as oxidase, a methanol oxidase is produced and preferably that methanol is used as inducing substrate,

4. Process according to claim 3, in which a methanol oxidase is produced that has the same amino acid sequence as the known methanol oxidase of Hansenula polymorpha CBS 4732, or a derivative of this sequence, obtained via enzyme engineering.

5. Process according to one or more of the preceding claims, in which the yeast is cultured in a nutritive medium that contains another source of carbon, chosen from the group consisting of sugars, such as glucose, sorbose, xylose, sorbitol, commerial available carbon sources such as molasses, and other carbon sources used in microbiology, such as glycerol and such like.

6. Process according to claim 5, in which methanol and glucose are used as sources of carbon in a molar ratio of methanol: glucose of (0.025-3):1, preferably (1-1.8):1.

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7. Process according to one or more of the preceding claims, in which the yeast is grown in a continuous fermentation.

8. Process according to one or more of the preceding claims, in which a yeast is used that is modified with the aid of recombinant DNA techniques or its progenity, in which the genetic information for the oxidase stands under control of a strong promotor, preferably a methanol oxidase (MOX) promotor, particularly that of the MOX of Hansenula polymorpha CBS 4732.

9. Process according to one of the preceding claims, in which the catalase-negative oxidase-containing yeast is, after finishing the fermentation, subjected to a drying treatment, whereby the oxidase enzyme is not inactivated, e.g. a freeze-drying treatment, or another treatment whereby the cells are made permeable or are inactivated.

10, Oxidase-containing, catalase-negative mutant of a yeast, obtained by making use of a process according to one or more of the claims 1-9.

11. Oxidase or an oxidase-containing composition, obtained by isolation of the oxidase from a yeast according to claim 10, optionally followed by processing the oxidase thus isolated to a composition with other components.

12. Use o-f an oxidase-containing, catalase-negative mutant of a yeast according to claim 10 or of an oxidase according to claim 11, together with a substrate for the oxidase, preferably ethanol, for
(1) the in situ production of hydrogen peroxide in a washing or bleaching process.,
(2) the qualitative and/or quantitative determination of a substrate for the oxidase, or
(3) the oxidation of a substrate of the oxidase within the framework of a chemical synthesis or of a process for purifying refuse.

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13. Washing or bleaching composition, characterized in that it contains an oxidase-containing, catalase-negative mutant of a yeast according to claim 10 or of an oxidase according to claim 11

Related papers:

Giuseppin, M.L.F., van Eijk, H.M.J., van Dijken, J.P. 1987. Production of a catalase-free oxidase and catalase-free oxidase containing yeast, and use thereof. WO87/07639.

Giuseppin, M.L.F., van Eijk, H.M.J. ,Verduyn, C., Bante, I., van Dijken, J.P., 1988, Production of catalase-free alcohol oxidase *by Hansenula polymorpha*. Appl. Microbiol. Biotechnol. **28**: 14-19.

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ABSTRACT

The enzyme methanol oxidase (MOX) can be used for many purposes on both small and large scale. MOX catalyses the reaction of alcohols, e.g. methanol and ethanol, with molecular oxygen to the corresponding aldehyde under formation of hydrogen peroxide. The many potential applications of MOX include the use of this enzyme for the generation of hydrogen peroxide in a bleach system for liquid detergents, the determination of alcohol concentrations, the cleansing of particular types of waste water, and many othersa dilution rate of 0.14 h^{-1} was optimal for MOX production by the wildtype strain in terms of enzyme-productivity and specific activity.

The methylotrophic yeast Hansenula polymorpha (CBS 4732) is a suitable source of this enzyme. Under optimal conditions this yeast can contain high levels of MOX of more than 37% of its cellular protein. This study was focussed on the optimization of MOX production using this yeast. For this purpose the studies were concentrated on a number of relevant aspects of the physiology and the fermentation of this yeast in order to find those factors that determine the net productivity of the production process. A simple formula for the productivity of the process was used to evaluate the effects of the various parameters which determine the process. The productivity can be described as the algebraic product of the dilution rate (D), the biomass concentration (X), the expression of the MOX-gene (E), the post-translational stability of MOX (S), and the efficiency of the recovery

The efficiency of the various steps in the biosynthesis of MOX was studied in continuous cultures to identify the rate-limiting production step (Chapter 2). In continuous cultures of H. polymorpha, grown on a mixture of methanol and glucose (1:4 w/w), it was found that many factors may determine the efficiency of the formation of active MOX. At low dilution rates it turned out that both the level of MOX-mRNA and the level of the cofactor (FAD) in MOX determined the activity of MOX in the cell. At high dilution rates (> 0.14 h^{-1}), the MOX activity decreased despite of the high MOX-mRNA levels, which is probably due to an increased breakdown of MOX-protein (see also Chapter 6). In the abovementioned experiments with a chosen ratio methanol/glucose of 1:4 (w/w) a dilution rate of 0.14h^{-1}

was optimal for MOX production by the wild-type strain in terms of enzyme-productivity and specific activity.

The removal of catalase is an expensive step in the down-stream processing of MOX. Therefore alternative routes for the production of MOX were studied. The purification could be simplified by using a catalase-negative mutant of H. polymorpha. It was necessary to determine the optimal cultivation conditions for this strain too, as it differs from that for the wild-type strain. Media and cultivation conditions suitable for the production of MOX were studied because this catalase-negative mutant is not able to grow on methanol as the sole carbon source. Efficient induction of MOX could be obtained in continuous cultures grown on mixtures of formaldehyde/glucose or formate/glucose. At a dilution rate of 0.1 h^{-1}, optimal induction of MOX was found at a molar ratio of 1.4 and 2.9, respectively (Chapter 3). The induction of MOX and the dissimilatory enzymes formaldehyde dehydrogenase and formate dehydrogenase in both the wild-type and the catalase-negative mutant showed similar patterns. The catalase-negative mutant grown under these conditions allowed production of MOX at lower costs. For some applications the purification can be reduced to a minimum by using the yeasts in a dried form.

Contrary to expectations the catalase-negative mutant was shown to be able to grow in continuous cultures on methanol/glucose mixtures, whereby relatively high levels of MOX activity were obtained. The optimal molar ratio was 1 to 2 mol methanol/mol glucose (Chapter 4). Indications of an alternative hydrogen-peroxide-decomposing enzyme system were found by exposing cultures of this strain to methanol. The detoxification capacity of this system was high: 2.6 and 3.9 mmol hydrogen peroxide per gram biomass per hour for a steady-state continuous culture and acontinuous culture after a methanol pulse, respectively. This detoxification system is most probably due to an increased level of cytochrome C peroxidase, which is located in the cristae of the mitochondria (Verduyn et al., to be published). This detoxification system will allow the industrial use of formaldehyde/glucose mixtures containing technically pure formaldehyde, which contains more than 10% methanol. This may reduce the material costs of the process. The detoxification system is only operational ín the living yeast and it is inactivated by drying the yeast. This means that no purification step is needed to remove the cytochrome C peroxidase. Thus whole dried cells can be used for the generation of hydrogen peroxide.

For a number of applications MOX has to be purified. This necessitates the break-up of the cells. In that case the strength of the cell wall is an important parameter. Therefore the dependence of the cell wall strength on the dilution rate and the substrate was studied. Cultures grown at a dilution rate below 0.1 h^{-1} showed thick and strong cell walls. Cells grown on methanol/glycerol mixtures or on methanol also produced cells with relatively thick and strong cell walls, which may hinder the break-up of the cells (Chapter 5). This means that the optimal dilution rate of 0.14 h^{-1} found for the wild-type strain, as described above, is also optimal with respect to this criterion.

A number of simplified mathematical models were developed and verified with experimental data in order to enable future studies on the optimization and simulation of growth of and MOX-production by H. polymorpha (Chapter b). This model describes growth and MOX production satisfactorily. It may be used to describe instabilities of cultures grown on methanol. In addition, the model describes the complex regulation of MOX synthesis in continuous cultures grown on methanol/glucose mixtures in terms of induction and repression. This induction and repression is effectuated by the residual concentration of respectively methanol and glucose. The induction was modelled by assuming an interaction of methanol (or its metabolites) and a repressor. The repression of MOX was ascribed to an induction of a proteolytic system, which was modelled by the interaction of glucose (or its metabolites) and a repressor. The induction and repression in this model form two counteracting mechanisms. These models may be used in further studies on scaling up and optimization of the fermentation process.

In connection with the studies described in Chapters 3 and 4, two patent applications were written for the application of the catalase-negative mutant in detergents. A summary of these patent applications has been included (Chapter 7).

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SAMENVATTING

Het enzym methanol oxidase (MOX) kan voor vele doeleinden gebruikt worden op zowel kleine als grote schaal. MOX katalyseert de reactie van alcoholen o.a. methanol en ethanol met moleculaire zuurstof tot het corresponderende aldehyde, waarbij waterstofperoxide wordt gevormd. De vele potentiële toepassingsmogelijkheden van MOX omvatten het gebruik van dit enzym voor de generatie van waterstofperoxide in een bleeksysteem voor vloeibare wasmiddelen, de bepaling van alcohol gehalten, het reinigen van bepaalde typen afvalwater en vele andere.

De methylotrofe gist Hansenula polymorpha (CBS 4732) is een geschikte bron voor dit enzym. Deze gist kan onder optimale kweekcondities hoge gehalten aan MOX bevatten tot meet dan 37% van het oplosbare cellulaire eiwit. Het onderhavige promotieonderzoek richtte zich op de optimalisering van de productie van MOX met deze gist. Hiertoe concentreerde de onderzoekingen zich op een aantal relevante aspecten van de fysiologie en de fermentatie van deze gist ten einde die factoren te vinden, die de netto-productiviteit van het MOX-productieproces bepalen. Ten einde de invloed van verschillende para-meters die het proces bepalen te kunnen evalueren werd een simpele formule voor de productiviteit van het proces werd gebruikt. Hierbij wordt de productiviteit bepaald door het algebraïsch product van de verdunningssnelheid (D), de biomassaconcentratie (X), de expressie van het MOX-gen (E), de post-translationele stabiliteit van MOX (S), en de efficiency van de opwerking.

De efficiency van de verscheidene stappen in de biosynthese van MOX werd onderzocht in continue culturen om de snelheidbepalende productiestap te identificeren (Hoofdstuk 2). In continue culturen van H. polymorpha, gekweekt op een mengsel van methanol en glucose (1:4 g/g), werd gevonden dat vele factoren de efficiency van de vorming van actief MOX kunnen bepalen. Bij lage verdunningsnelheden bleken zowel het niveau van de MOX-mRNA als het gehalte aan de cofactor (FAD) in MOX de activiteit van MOX in de cel te bepalen. Bij hoge verdunningsnelheden (> 0.14 h^{-1}) bleek de MOX activiteit ondanks de hoge MOX-mRNA niveaus af te nemen, hetgeen waarschijnlijk te wijten is aan een verhoogde afbraak van MOX-eiwit (zie ook Hoofdstuk 6). In de voorafgaande experimenten bij een gekozen verhouding methanol / glucose van 1:4 (g/g) bleek de verdunningssnelheid 0.14 h^{-1} te leiden tot een optimale MOX-productie in termen van enzym-productiviteit en specifieke activiteit.

In de opwerking van MOX bleek de verwijdering van catalase een kostbare processtap. Daarom werden alternatieve routes bestudeerd voor de productie van MOX. De opwerking kon worden verbeterd door gebruik te maken van een catalase-negatieve mutant van H. polymorpha. Omdat deze stam anders is dan de wild-type stam, werden hiervoor eveneens de optimale kweekcondities bepaald. Voor de inductie van MOX in deze stam werden geschikte media en kweekcondities onderzocht, daar deze mutant zonder het catalase niet op methanol als enige koolstofbron kan groeien. Efficiënte inductie van MOX kon worden verkregen in continue culturen gekweekt op mengsels van formaldehyde en glucose of formiaat en glucose. In deze mengsels werd bij een verdunningsnelheid van 0.1 h^{-1} een optimale inductie van MOX gevonden bij molaire ratio's van respectievelijk 1.4 en 2.9 mol/mol (Hoofdstuk 3). De inductie van MOX en de dissimilatieve enzymen formaldehydedehydrogenase en formiaat dehydrogenase in de wild-type en de catalase-negatieve mutant vertoonden een sterke gelijkenis. De aldus gekweekte catalase-negatieve mutant maakt het mogelijk om MOX te maken bij lagere productie kosten. Voor sommige applicaties kan de opwerking tot een minimum worden beperkt en kunnen de cellen in gedroogde vorm worden gebruikt.

Tegen de verwachting in bleek de catalase-negatieve mutant te kunnen groeien in continue culturen op methanol/glucose mengsels, waarbij een relatief hoge MOX activiteit werd bereikt. De optimale molaire ratio bedroeg 1 tot 2 mol/mol (Hoofdstuk 4). Door cultures van deze stam bloot te stellen aan methanol werden aanwijzingen gevonden voor een alternatief waterstofperoxide afbrekend enzymsysteem. De ontgiftings capaciteit van dit systeem was hoog en bedroeg 2.6 en 3.9 mmol waterstofperoxide per gram biomassa per uur voor respectievelijk een steady-state continue cultuur en een continue cultuur na een methanol puls. Dit ontgiftingssysteem is waarschijnlijk een verhoogd niveau van cytochroom C peroxidase, dat gelocaliseerd is in de cristae van de mitochondriën (Verduyn et al., 1987). Het ontgiftingssysteem maakt het mogelijk om voor industriële toepassingen formaldehyde/glucose meng-sels te gebruiken met tech-

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nisch zuiver formaldehyde, dat meer dan 10% methanol kan bevatten. Dit kan een besparing opleveren in de grondstofkosten. Het ontgiftingsysteem is alleen operationeel in levende cellen en wordt onmiddellijk geïnactiveerd bij het drogen van de gist. Dit betekent dat geen zuiveringstap nodig is om deze cytochroom C peroxidase activiteit te verwijderen. Zodoende kunnen gedroogde cellen reeds gebruikt worden voor waterstofperoxide-productie.

Voor een aantal toepassingen dient MOX gezuiverd te worden. Daartoe moeten de cellen van H. polymorpha worden opengebroken. De celwandsterkte is hierbij een belangrijk gegeven. Daarom werd de afhankelijkheid van de celwandsterkte van de verdunningsnelheid en het gebruikte substraat bestudeerd. Cultures gekweekt bij een verdunning snelheid lager dan 0.1 h^{-1} vertoonden dikke en moeilijk of te breken celwanden. Evenzo leverden cultures, gekweekt op methanol/glycerol mengsels of op methanol, cellen met relatief dikke en sterke celwanden, hetgeen het opbreken van de cellen kan bemoeilijken (Hoofdstuk 5). Dit betekent dat voor de wild-type stam de eerder genoemde optimale ver- dunningsnelheid van 0.14 h^{-1} ook ten aanzien van deze randvoorwaarde geschikt lijkt.

Ten behoeve van toekomstige studies aan optimalisatie en simulatie van de groei van en de MOX-productie door H. polymorpha werden enkele vereenvoudigde mathematische modellen ontwikkeld en getoetst aan de hand van literatuurgegevens en nieuwe experimentele gegevens (Hoofdstuk 6). Dit model bleek de groei en MOX-productie voldoende te kunnen beschrijven. Zo konden instabiliteiten van culturen op methanol worden gesimuleerd. Daarnaast werd met dit model de complexe regulatie van de MOX-synthese in continue culturen gekweekt op methanol/glucose beschreven in termen van inductie en repressie als gevolg van respectievelijk de residuele methanol en glucose concentratie. De inductie werd gemodelleerd aan de hand van een interactie van methanol (of zijn metabolieten) met een repressor. De repressie van MOX werd in het model toegeschreven aan de inductie van een proteolytische systeem. Dit werd beschreven door een interactie van glucose (of zijn metabolieten) met een repressor. De inductie en repressie in dit model vormen op deze wijze twee tegen elkaar inwerkende mechanismen. Deze modellen kunnen worden gebruikt als basis voor verder onderzoek aan opschaling en optimalisatie van het fermentatieproces.

Naar aanleiding van de resultaten in hoofdstuk 3 en 4 werden twee octrooiaanvragen opgesteld, die de productiewijze van catalase-vrije MOX beschrijven. Voor de volledigheid zijn de samenvattingen van deze octrooiaanvragen in Hoofdstuk 7 opgenomen.

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