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Development of Amine Oxidase-Containing Peroxisomes in Yeasts During Growth on Glucose in the Presence of Methylamine as the Sole Source of Nitrogen

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Abstract. The metabolism of methylamine as the nitrogen source for growth of the non-methylotrophic yeast *Candida utilis* and the methylotrophic yeast *Hansenula polymorpha* was investigated. Growth of both organisms in media with glucose and methylamine was associated with the presence of an amine oxidase in these cells. The enzyme catalyses the oxidation of methylamine by molecular oxygen into ammonia, formaldehyde and hydrogen peroxide and it is considered to be the key enzyme in methylamine metabolism in the organisms studied.

In addition to synthesis of amine oxidase, derepression of catalase, formaldehyde and formate dehydrogenase was also observed upon transfer of cells of the two organisms from media containing ammonium ions into media containing methylamine as the nitrogen source.

The synthesis of enzymes was paralleled by the development of a number of large microbodies in the cells. Cytochemical staining experiments indicated that the amine oxidase activity was located in the microbodies in both organisms. Catalase-activity was also demonstrated in these organelles, which can therefore be considered as peroxisomes. The present contribution is the first description of a peroxisomal amine oxidase.

Key words: *Candida utilis* – *Hansenula polymorpha* – Amine oxidase – Methylamine – Nitrogen metabolism – Peroxisome.

The utilization of methylated amines as the sole nitrogen source by yeasts was first described by van der Walt (1962), and has since been investigated mainly for taxonomic reasons (Brady, 1965; La Rue and Spencer, 1968). Recently it was found that the ability to utilize methylated amines (methylamine in particular) is widely distributed among yeasts. It is particularly common in the genera *Candida*, *Pichia* and *Hansenula* (van Dijken and Bos, pers. comm.). It has been suggested (van der Walt, 1962; Yamada et al., 1965; Yamada et al., 1966), that the metabolism of amines in fungi is identical to that in animal cells in which a hydrogen peroxide producing oxidase is involved which catalyses the following reaction:

$$RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + NH_3 + H_2O_2 .$$

The synthesis and properties of amine oxidase in the fungus *Aspergillus niger* has been studied extensively by Yamada et al. (1965a, b, c, and d) after growth of the organism in media with mono- and diamines. They observed an optimum in enzyme activity in the mycelium during the early logarithmic growth phase and a subsequent decrease in activity after prolonged cultivation. The same pattern of amine oxidizing activity was found in *Candida* sp. WY-3 cells during growth on n-methyl-n-butylamine and trimethylamine (Yamada et al., 1976).

Little is known about the localization of amine oxidase activity in fungi. In mammalian cells monoamine oxidase is known to be located on the outer membrane of mitochondria, and is moreover, considered as a marker enzyme for outer mitochondrial membranes (Lloyd, 1974). In the protozoan *Tetrahymena pyriformis* monoamine oxidase activity was found to be located predominantly in the mitochondrial pellet (Feldman et al., 1977). In the yeast *Candida* sp. WY-3, grown on amines, Yamada et al. (1976) suggested that the amine oxidation activity might be located in an organelle. In the course of our studies on the development and physiological function of peroxisomes in yeasts, we observed that large microbodies were present in cells of both the methylotrophic yeast *Hansenula polymorpha* and in the non-methylotrophic yeast *Candida utilis* after growth on

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glucose in the presence of methylamine as the sole source of nitrogen. These observations led us to investigate the metabolism of methylamine in yeast. The results, presented in this paper, showed that in cells of these organisms, when grown on glucose and methylamine as the sole nitrogen source, a number of large peroxisomes develop, which contain amine oxidase and catalase.

Materials and Methods

Microorganisms and Cultivation. *Hansenula polymorpha* de Morais et Maya CBS 4732 and *Candida utilis* NCYC 321 were grown in 2 l shake flasks at 37°C and 29°C respectively, in the mineral medium of van Dijken et al. (1976). Before transfer into media with methylamine as the sole nitrogen source, the cells were precultured 3 times in media containing 0.25% glucose and 0.25% ammonium sulphate as described before (Veenhuis et al., 1979). Cells, grown in this way were harvested at $OD_{663} = 1.0$, washed twice in 200 ml mineral medium without carbon- and nitrogen source and subsequently transferred into a medium containing 0.25% glucose and 0.25% methylamine-hydrochloride as the nitrogen source, to an OD_{663} of 0.1.

Preparation of Spheroplasts. Spheroplasts were prepared by treatment of suspensions of whole cells with "Zymolyase" (Kitamura et al., 1971), for 5 – 30 min at 37°C according to the procedure of Osumi et al. (1975). The pretreatment of cells with mercaptoethanol was omitted since this compound inhibited amine oxidase activity.

Enzyme Assays. The preparation of cell-free extracts and the estimation of alcohol oxidase catalase, formaldehyde dehydrogenase and formate dehydrogenase were as described previously (van Dijken, 1976). Methylamine oxidase activity was assayed in the same manner as described for alcohol oxidase, except that 50 mM sodium phosphate buffer pH 8.0, and 1 mM methylamine as substrate were used. Enzyme activities were determined at 37°C for *H. polymorpha* and at 29°C for *C. utilis*.

Determination of Formate. Formate was determined according to the method of Lang and Lang (1972).

Cytochemical Staining. Cytochemical staining procedures for the detection and localization of catalase and oxidase activities were performed as described previously (Veenhuis et al., 1976). In the case of amine oxidase, 20 mM substrate was used.

Freeze Etching. Cells were incubated for 10 min in 15% (w/v) glycerol, frozen in FREON and freeze fractured in a Balzer's freeze-etch unit, according to the method described by Moor (1964).

Fixation and Postfixation. Whole cells were fixed with 1.5% $KMnO_4$ for 20 min at room temperature. Spheroplasts were prefixed for 15 min in 6% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 at 0°C, and postfixed for 45 min in a solution of 2.5% $K_2Cr_2O_7$ and 1% OsO_4 in 0.1 M cacodylate buffer, pH 7.2. After dehydration in a graded ethanol series the cells were embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Philips EM 300 or EM 201.

Quantitative Analysis of Thin Sections. The average number of peroxisomes was estimated by random counting of cell profiles in thin sections (Veenhuis et al., 1978). The volume density of peroxisomes in the cytoplasm was estimated with the point counting technique according to Weibel and Bolender (1976). Student's *t*-test was used for statistical analysis.

Results

Biochemical Experiments
Induction of Enzymes Involved in Methylamine Metabolism

Candida utilis and *Hansenula polymorpha* were both unable to grow in chemically defined media with methylamine as the only carbon source. Both organisms were, however, able to utilize this compound as the only nitrogen source for growth. During growth in the presence of methylamine, amine oxidase was detected in cell-free extracts of the two organisms. The activity of this enzyme was measured using an oxygen

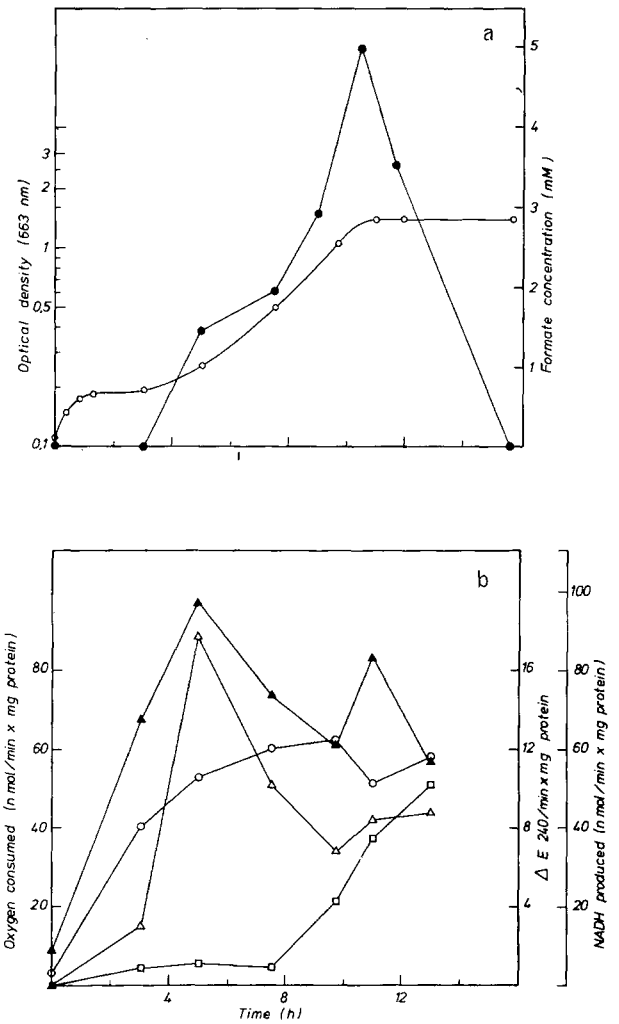


Fig. 1 A and B. Growth and enzyme activities of *Candida utilis* in media with glucose as carbon- and methylamine as sole nitrogen source. **A** Growth (○) expressed as optical density at 663 nm, formate concentration in the medium expressed in mM (●). **B** Amine oxidase (△), formaldehyde dehydrogenase (▲), formate dehydrogenase (□) and catalase activity (○). Oxidase activity expressed as nmol $O_2 \times min^{-1} \times mg\ protein^{-1}$, formaldehyde dehydrogenase and formate dehydrogenase activity as nMol NADH $\times min^{-1} \times mg\ protein^{-1}$ and catalase activity as $\Delta E_{240} \times min^{-} \times mg\ protein^{-1}$

Table 1. Enzyme activities in cell-free extracts of *Hansenula polymorpha* grown in media with glucose and ammonium sulphate after the transfer of cells into media with glucose and methylamine as the nitrogen source, harvested in the mid-exponential-($OD_{663} = 1.0$) and the stationary growth phase ($OD_{663} = 2.2$)

	I	II	III	IV	V
Glucose/$(NH_4)_2SO_4$	0	0	0.7	126.5	0
Glucose/CH_3NH_2 ($OD_{663} = 1.0$)	11.1	0	12.4	367.1	0
Glucose/CH_3NH_2 ($OD_{663} = 2.2$)	10.4	28.7	12.3	513.8	114.2

I = Amine oxidase (nmol × min^{-1} × mg protein^{-1}); II = alcohol oxidase (nmol × min^{-1} × mg protein^{-1}); III = catalase (ΔE_{240} × min^{-1} × mg protein^{-1}); IV = formaldehyde dehydrogenase (nmol × min^{-1} × mg protein^{-1}); V = formate dehydrogenase (nmol × min^{-1} × mg protein^{-1})

Table 2. Effect of the nature of the nitrogen source (0.25%) on the specific growth rate of *C. utilis* and *H. polymorpha*, grown in chemically defined media supplemented with 0.25% glucose

Nitrogen source	Specific growth rate (h^{-1})	
	C. utilis	*H. polymorpha*
0.25% $(NH_4)_2SO_4$	0.55	0.46
0.25% CH_3NH_2	0.28	0.23
0.25% $(NH_4)_2SO_4$ and 0.25% CH_3NH_2	0.55	0.46

electrode system; enzyme activity was not influenced by dialyzing the crude cell-free extracts or by including phenazine methosulphate (1 mM final concentration) in the reaction mixture. The kinetics observed indicated that the enzyme possesses a fairly high K_m for oxygen, similar to that of alcohol oxidase of *H. polymorpha* (van Dijken et al., 1976).

The behaviour of *C. utilis* cells, grown on glucose and ammonium sulphate and transferred into a medium with glucose and 0.25% methylamine as the sole source of nitrogen, is shown in Fig. 1A. Exponential growth started 3 h after the transfer and was accompanied by the accumulation of formate in the medium. The formate concentration reached a maximum of 5 mM in the late exponential growth phase. In the stationary growth phase its concentration gradually decreased to zero.

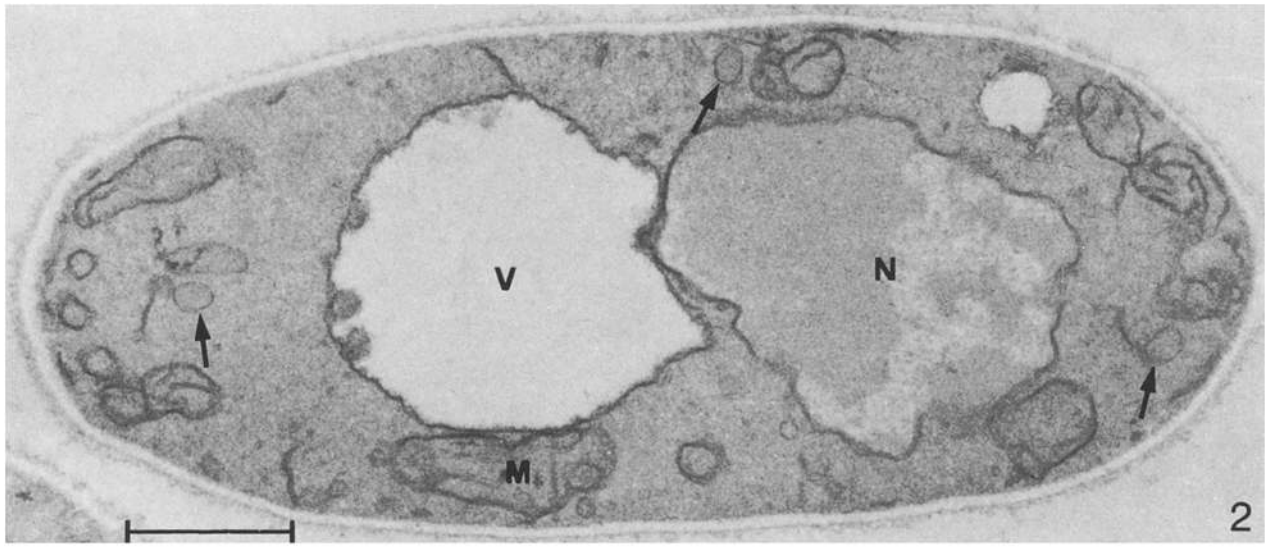
Synthesis of amine oxidase started immediately after the transfer to the methylamine-containing media. The activity of the enzyme increased drastically during early exponential growth and reached a maximum in the mid-exponential growth phase (Fig. 1B). A similar pattern was observed for catalase and formaldehyde dehydrogenase activity. Furthermore, the activity of formate dehydrogenase was low immediately after the transfer of the cells, while a sharp increase in the activity of this enzyme was observed between the mid-exponential and stationary growth phase (Fig. 1B).

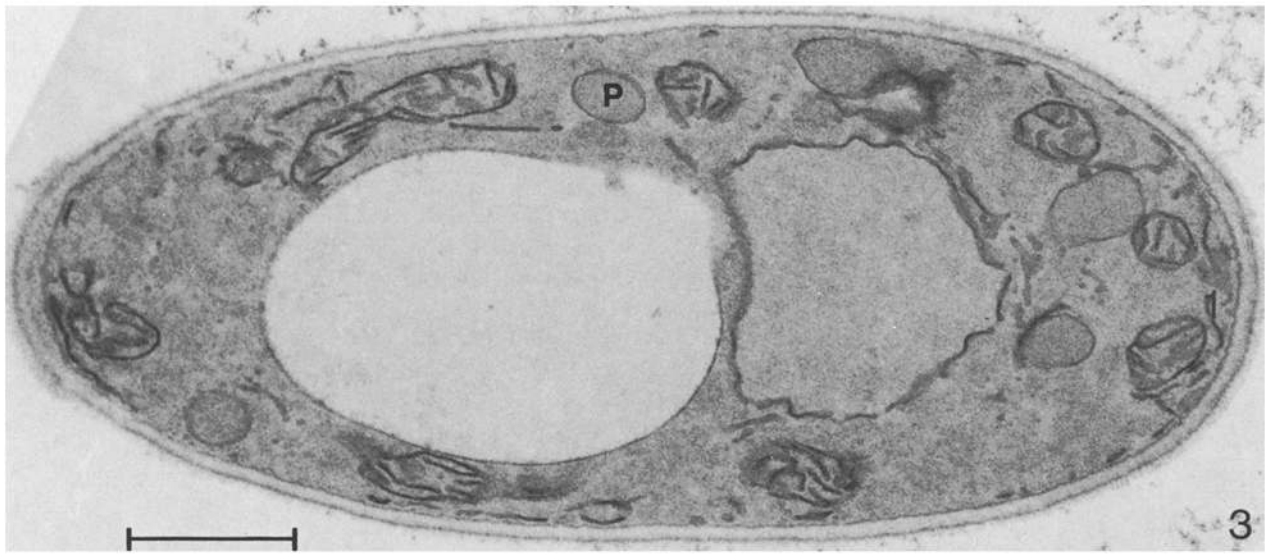
Enzyme profiles in *H. polymorpha*, transferred from glucose/ammoniumsulphate to glucose/methylamine media, were similar to those described for *C. utilis*. However, the maximum activities of all enzymes concerned except for catalase were lower in *H. polymorpha* (Table 1). As in *C. utilis*, exponential growth on glucose and methylamine was associated with the excretion of formate into the growth medium. In contrast to *C. utilis*, in *H. polymorpha* the synthesis of formate dehydrogenase was repressed during the first hours of growth and started in the late-exponential growth phase. Furthermore, as in the experiments of Eggeling and Sahm (1978), the synthesis of alcohol oxidase became derepressed in the late-exponential growth phase. This enzyme was not detected in cell-free extracts of *C. utilis*.

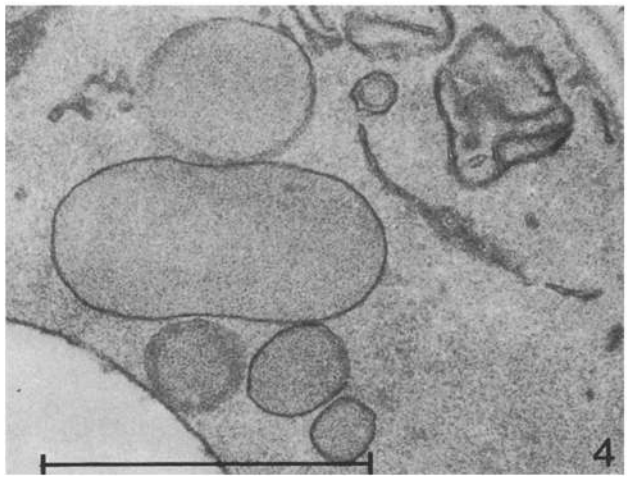
Although the growth rate of both organisms in media with methylamine as the nitrogen source was significantly lower than in media with ammonium sulphate (Table 2), there was hardly any influence on the cell yield of both organisms.

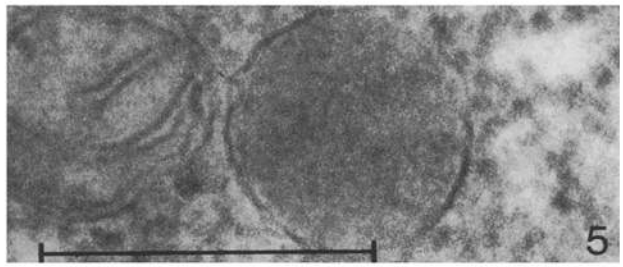
Electron Microscopical Observations
Cell Morphology and Ultrastructure

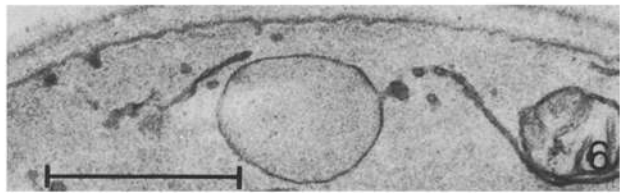
The transfer experiments discussed above were performed with cells grown into the mid-exponential growth phase in media with glucose and ammonium sulphate ($OD_{663} = 1.0$). Cells of *C. utilis*, grown in this way, contained besides the usual cell organelles such as nucleus, mitochondria, ER and vacuole, a number of small microbodies, as was revealed in thin sections of $KMnO_4$-fixed cells (Fig. 2). The dimensions of these organelles varied from 0.2–0.5 μm. The microbodies were scattered throughout the cytoplasm, irregular of shape and frequently associated with strands of ER. After transfer into glucose/methylamine media, a number of large microbodies developed in the cells during exponential growth, most probably by growth of the organelles present in the inoculum cells (Fig. 3). As in cells grown in the glucose ammonium sulphate-containing media the organelles were scattered throughout the cytoplasm, but also clusters of microbodies were observed (Fig. 4). The matrix of these organelles never showed crystalline inclusions (Fig. 5). Considerable differences between individual microbodies present in one cell were observed; they varied in shape from rounded to highly elongated (Fig. 4, 7a–f), and in dimensions of 0.5–1 μm. In freeze-etch preparations the membranes surrounding the microbodies in *C. utilis* cells, showed a smooth appearance (Fig. 7). A similar structure which may be characteristic











Figs. 2–6

for peroxisomes, has also been observed in cells of *H. polymorpha* (Veenhuis et al., 1978; 1979), Serial sections revealed that the microbodies were in close proximity to the mitochondria and to strands of ER (Figs. 6, 7a – f). The serial sections also demonstrated the presence of a giant, branched mitochondrion in the cells, as was also frequently observed in freeze-etch preparations (Fig. 9).

The results of the transfer experiments, performed with cells of *H. polymorpha*, were rather similar to the results described above for *C. utilis*. The ultrastructure of *H. polymorpha* cells, grown into the mid-exponential growth phase in glucose/ammonium sulphate media, has been described previously (Veenhuis et al., 1979), and the cells contain besides the usual cell organelles, generally one small peroxisome (0.1 – 0.2 μm), irregular of shape, and located against the cell wall (Fig. 10). After the transfer, during mid-exponential growth in the glucose/methylamine medium, the microbodies in these cells increased in size and subsequently in number by means of division of small organelles from the one originally present. Crystalline inclusions were not observed in the matrix of the organelles. In contrast to the clusters of peroxisomes developing in methanol-growing cells (Veenhuis et al., 1979), the organelles were generally scattered throughout the cytoplasm (Fig. 11). Frequently close associations with strands of ER and mitochondria were observed. In the late-exponential growth phase, when alcohol oxidase activity appeared in the cells, crystalline inclusions became apparent in the peroxisomal matrix. However, the organelles remained irregular of shape. The cubical shaped peroxisomes, which are so characteristic in stationary methanol-grown cells (Veenhuis et al., 1978), were not observed.

The development of peroxisomes in the cells of both organisms after the transfer into glucose/methylamine media, was also followed by morphometrical techniques (Table 3). The results clearly support the above observations in that apart from an increase in volume density caused by an increase in number of microbodies the individual organelles also had increased in size. However, a remark has to be made with respect to the increase in the number of peroxisomes in cells of *C. utilis*. The numbers given are the average numbers of peroxisomal profiles observed in thin sections of cells of this organism. It will be clear that an increase in size of an individual organelle also increases the chance of cutting a profile. Serial sections revealed that in glucose/ammoniumsulphate-grown cells of *C. utilis* an average of 12 microbodies was present, while in the stationary phase cells of this organism, grown in glucose/methylamine media this number had increased to 16 per cell. Therefore, in *C. utilis* the increase in volume density is mainly due to growth of individual organelles already present. This is in contrast to the response observed in *H. polymorpha*; in this organism the inoculum cells generally contained only one peroxisome while in stationary-phase cells grown in glucose/methylamine media generally 5 per cell were observed.

Table 3. Number and volume density of peroxisomes of *C. utilis* and *H. polymorpha*, grown on glucose and ammonium sulphate, from the midexponential growth phase ($OD_{663} = 1.0$), and after the transfer of these cells into glucose and methylamine as the nitrogen source. Cells grown on glucose/methylamine were harvested in the mid-exponential growth phase ($OD_{663} = 1.0$) and in the stationary phase ($OD_{663} = 1.45$ for *C. utilis* and $OD_{663} = 2.2$ for *H. polymorpha*). The number of peroxisomes is given as the average number per section. The volume density is expressed as percentage of the cytoplasmic volume

Growth conditions	*C. utilis*		*H. polymorpha*	
	Number	Volume	Number	Volume
Glucose/$(NH_4)_2SO_4$ mid-exponential	0.3	0.15	0.04	0.09
Glucose/CH_3NH_2 mid-exponential	0.7	3.0	0.4	2.3
Glucose/CH_3NH_2 stationary	2.7	9.3	0.9	5.2

Electron micrographs. Abbreviations: *CM* cell membrane; *L* lipoid droplet; *M* mitochondrion; *N* nucleus; *P* peroxisome; *V* vacuole. Cells were fixed with $KMnO_4$, unless otherwise stated. The marker represents 1.0 μm. In the freeze-etch electron micrographs the arrow indicates the direction of shadowing

Figs. 2—9 represent electron micrographs of *Candida utilis*

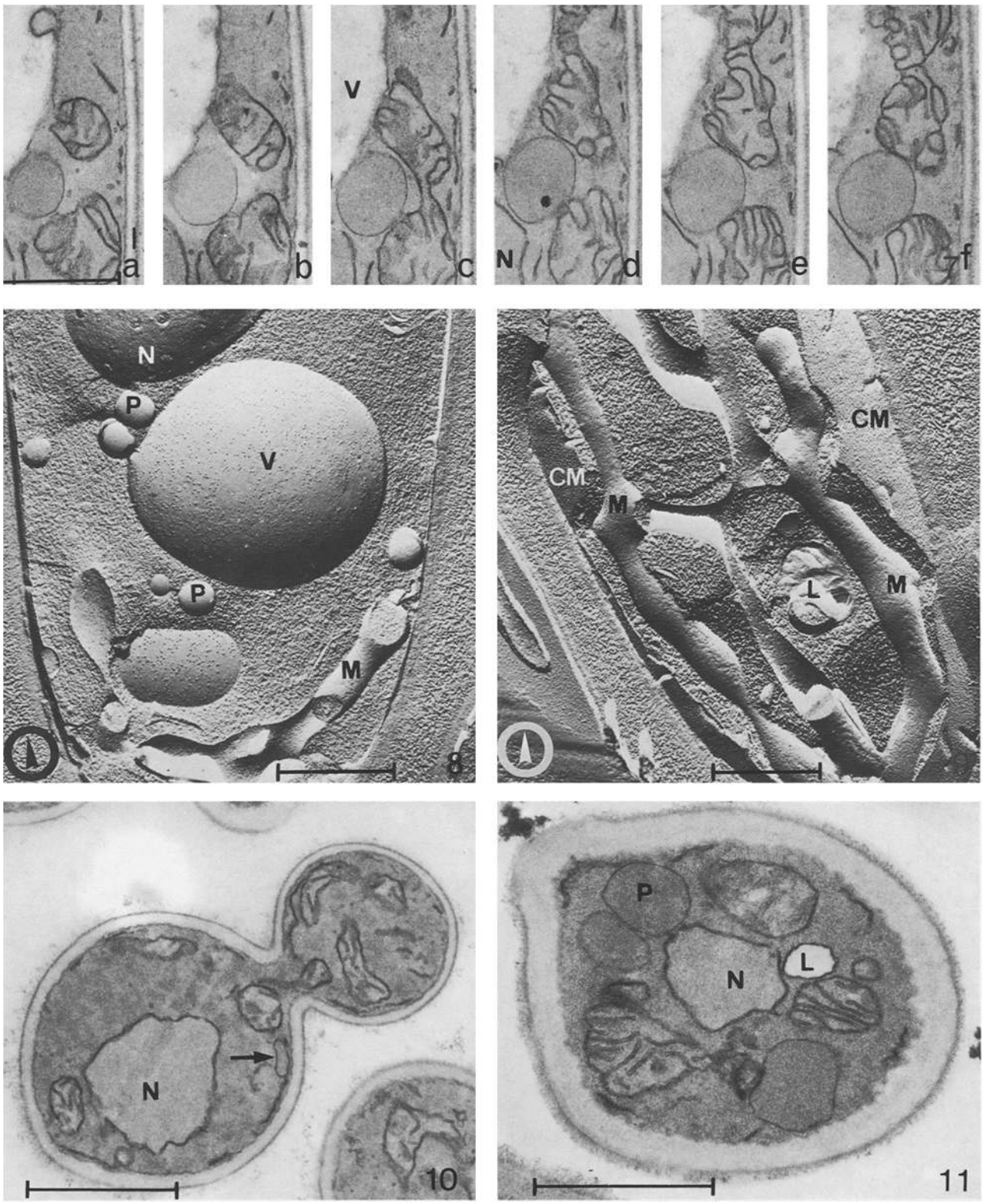
Fig. 2. Section through a cell grown on glucose/ammonium sulphate from the mid-exponential growth phase, showing several small peroxisomes (*arrows*)

Fig. 3. Section through a cell grown on glucose/methylamine from the mid-exponential growth phase, showing a number of large peroxisomes scattered throughout the cytoplasm

Fig. 4. Detail of a cell grown on glucose/methylamine showing a cluster of large peroxisomes

Fig. 5. Detail of a cell grown on glucose/methylamine from the stationary phase, showing the homogeneous structure of the peroxisomal matrix after fixation with glutaraldehyde-$OsO_4/K_2Cr_2O_7$

Fig. 6. Detail of a cell grown on glucose/methylamine to illustrate the close relationship between peroxisomes and the endoplasmic reticulum



Figs. 7–11

Cytochemical Staining Experiments

Cytochemical staining experiments have been performed on whole cells or on spheroplasts, prefixed in ice-cold glutaraldehyde. Since the techniques for demonstrating oxidase activities used in our laboratory are based on the chemical or enzymatic trapping of H_2O_2 generated by the oxidases, we first examined the effect of exposing the cells to glutaraldehyde on the capacity of the cells to oxidize excess methylamine. The results, shown in Fig. 12, reveal that this capacity of whole cells, gradually decreased with increasing fixation time. On the basis of these results, we decided to perform the cytochemical experiments on cells prefixed for 15 min.

Both cytochemical staining techniques for demonstrating amine oxidase activity, namely the direct method, involving aerated incubations of spheroplasts with $CeCl_3$ and methylamine in the presence of aminotriazole to inhibit catalase activity, as well as the indirect technique in which whole cells are incubated with DAB and methylamine, resulted in positively stained peroxisomes (Figs. 13–15). The presence of catalase in these organelles was demonstrated on the basis of incubations of spheroplasts with DAB and exogenous H_2O_2, which also showed positively stained peroxisomes (Fig. 16). Apart from the staining of peroxisomes, staining deposits were also observed in the mitochondria, both after incubations in the presence of $CeCl_3$ and DAB. Incubations with DAB and methylamine, performed in the presence of 1 mM KCN in order to inhibit staining of mitochondria surprisingly did not lead to staining of peroxisomes. Biochemical experiments showed that this was due to the fact that the oxidation of methylamine in both whole cells and in cell-free extracts, was completely inhibited by the presence of low concentrations of KCN.

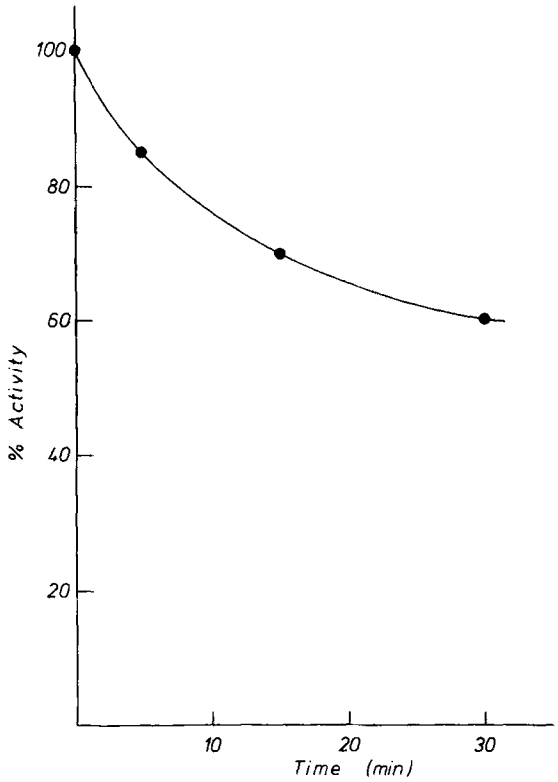


Fig. 12. Effect of fixation with 6% glutaraldehyde on the capacity of whole cells of *Candida utilis* to oxidize excess methylamine

Discussion

Yeasts are generally unable to grow in media containing methylated amines as the sole source of carbon and energy (van der Walt, 1962; Brady, 1965; La Rue and Spencer, 1968). However, several species can utilize these compounds as the sole source of nitrogen for growth (Bos and van Dijken, pers. comm.). Our experiments with *Candida utilis* and *Hansenula polymorpha* clearly indicated that growth of these organisms in media with glucose and methylamine is associated with the synthesis of an O_2-dependent, H_2O_2-producing oxidase. This enzyme catalyses the conversion of methylamine into ammonia, formaldehyde, and H_2O_2 and is probably the key enzyme in nitrogen metabolism during growth of these yeasts on methylamine. During exponential growth of *C. utilis* and *H. polymorpha* on glucose and methylamine, the growth rate is presumably determined by the rate of oxidation of the amine. The growth rate is considerably lower in media with methylamine (Table 2), which may be due to the fairly low activity of amine oxidase in cell-free extracts of the two organisms (Fig. 1; Table 1). Growth

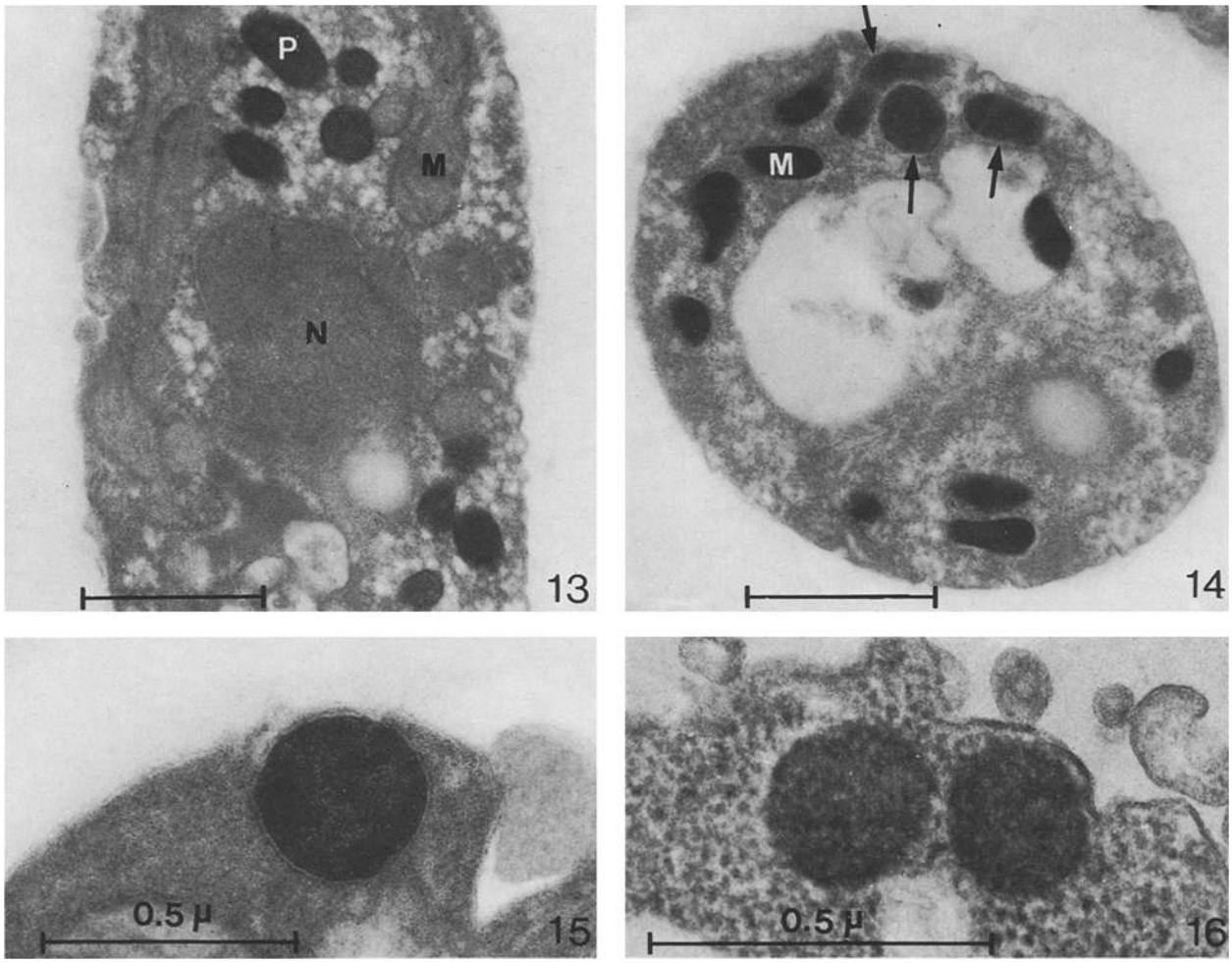
Fig. 7a—f. Serial sections through a cell grown on glucose/methylamine, to demonstrate the close relationship of an elongated peroxisome and the mitochondrion

Fig. 8. Detail of a freeze-fractured cell, grown on glucose/methylamine, to illustrate the smooth surface of the peroxisomal membrane

Fig. 9. Part of a freeze-fractured cell, showing the large branched mitochondrion

Fig. 10. Section through a cell of *Hansenula polymorpha* grown on glucose/ammonium sulphate from the exponential growth phase, showing one small peroxisome (*arrow*)

Fig. 11. Section through a cell of *Hansenula polymorpha* grown on glucose/methylamine, from the stationary phase, in which a number of large peroxisomes are seen



Figs. 13–16. Illustrations of cytochemical staining experiments performed with glutaraldehyde-fixed spheroplasts of cells grown in glucose/methylamine containing media

Figs. 13 and 14. Represent cells of *Candida utilis*, showing positively stained peroxisomes after incubation with $CeCl_3$ and methylamine (Fig. 13) and after incubation with DAB and methylamine (Fig. 14). After incubations including DAB, the mitochondria are also heavily stained (Fig. 14)

Figs. 15 and 16. Details of cells of *Hansenula polymorpha*, showing positively stained peroxisomes after incubation with $CeCl_3$ and methylamine (Fig. 15) and after incubation with DAB and H_2O_2 (Fig. 16)

on methylamine as the nitrogen source did not lead to an increase in growth yield. This was rather unexpected since the oxidation of formaldehyde to CO_2 leads to the production of reduced NAD. The reason for this observation is not clear, but may be associated with an energy-dependent uptake system for methylamine similar to that reported by Roon et al. (1975) in *Saccharomyces cerevisiae*.

Apart from the synthesis of amine oxidase, growth of the yeasts in media with glucose and methylamine was also associated with derepression of the catabolic enzymes formaldehyde and formate dehydrogenase, catalase and, in case of *H. polymorpha*, alcohol oxidase. These enzymes play an important role in the metabolism of methanol in methylotrophic yeasts (van Dijken et al., 1976; Sahm, 1977; Tani et al., 1978). Eggeling and Sahm (1978) studied the regulation of these enzymes in *H. polymorpha* during growth on glucose in batch cultures and showed that the synthesis of alcohol oxidase, formaldehyde and formate dehydrogenase was fully repressed during exponential growth, while catalase was present to a very low extent. However, in the stationary growth phase, when glucose was exhausted, a rapid increase in activity of these enzymes was observed. The regulation of these enzymes was also studied in glucose-limited chemostat cultures of *H. polymorpha* and *Kloeckera* sp. 2201 (Egli et al., 1980). In both organisms derepression of alcohol oxidase, catalase and formaldehyde and formate dehydrogenases was observed as the growth rate was

decreased, which was due to a lowered glucose concentration.

In contrast, in our batch culture experiments with *H. polymorpha* we observed a rapid increase in both catalase and formaldehyde dehydrogenase activity, immediately after the transfer from ammonium sulphate to methylamine as the nitrogen source, in the presence of high glucose concentrations. We therefore suggest that it is likely that the presence of methylamine as the nitrogen source can release the repression of catalase and formaldehyde dehydrogenase, but not the repression of alcohol oxidase and formate dehydrogenase, indicating that the synthesis of these enzymes is probably regulated separately.

Electron microscopic observations revealed that during growth of *C. utilis* and *H. polymorpha* in media with glucose and methylamine a number of large microbodies developed in the cells. On the basis of the cytochemical staining experiments, which indicated the presence of amine oxidase together with catalase in these organelles, they can be regarded as peroxisomes, according to the definition of de Duve and Baudhuin (1966). In *H. polymorpha* these peroxisomes originated by a process of growth and division of the small organelle originally present in the glucose/ammonium sulphate grown cells; this process is similar to that described for the development of peroxisomes in cells transferred from glucose to methanol-containing media (Veenhuis et al., 1978, 1979). Identical observations were made in cells of *C. utilis*. However, in contrast to *H. polymorpha* a small increase in number of peroxisomes per cell occurred as was revealed in serial sections. We did observe budding of small organelles from mature ones. This possibly explains the presence of small clusters of organelles in the cells, a phenomenon not observed in the inoculum cells.

In mammalian cells the cytochemical localization of mono amine oxidase has been performed using an artificial electron acceptor (Boadle and Bloom, 1968). These results, supported by biochemical evidence, indicated that mono amine oxidase is present in the outer membranes of the mitochondria and have led to the view that this enzyme may be regarded as a marker enzyme for the mitochondrial outer membranes (Lloyd, 1974). Efforts to localize mono amine oxidase in cells of *S. cerevisiae* have failed. In this connection it is of interest to note that several strains of this organism are incapable of using methylated amines as a nitrogen source for growth (Brady, 1965; La Rue and Spencer, 1968), which may explain the negative results obtained.

The results of our cytochemical experiments did not exclude the presence of amine oxidase activity in the mitochondria, since both after incubations with DAB or $CeCl_3$ – in the presence of methylamine as a substrate – staining deposits were also observed in the mitochondria. Staining deposits in the mitochondria after incubations in the presence of DAB or $CeCl_3$ are well-known and are due to action of the cytochrome C-cytochrome oxidase complex (van Dijken et al., 1975; Veenhuis et al., 1976). However, we were unable to discriminate between amine oxidase and cytochrome oxidase activity, since CN^--ions, generally used to block cytochrome activity, also completely inhibited the amine oxidase activity. In addition, prolonged fixation with glutaraldehyde in order to destroy cytochrome *c* peroxidase activity (Roels et al., 1975) could not be used, since apart from the inactivation of this enzyme the activity of the amine oxidase also largely disappeared. The properties of yeast amine oxidase have not been adequately studied. However, a peroxisomal amine oxidase has not been described before and can now be added to the gradually expanding list of peroxisomal enzymes. On the basis of our results studies on the localization of amine oxidase in various organisms require very careful examination. Furthermore the role of peroxisomes in nitrogen metabolism and its regulation in yeast – and possibly other organisms – offers an interesting scope for future investigations.

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References

Boadle, C. M., Bloom, F. E.: A method for the fine structural localization of monoamine oxidase. J. Histochem. Cytochem. **17**, 331–340 (1968)

Brady, B. L.: Utilization of amino compounds by yeasts of the genus *Saccharomyces*. Antonie van Leeuwenhoek J. Microbiol. Serol. **31**, 92–105 (1965)

Van Dijken, J. P., Veenhuis, M., Vermeulen, C. A., Harder, W.: Cytochemical localization of catalase activity in methanol-grown *Hansenula polymorpha*. Arch. Microbiol. **105**, 261–267 (1975)

Van Dijken, J. P., Otto, R., Harder, W.: Growth of *Hansenula polymorpha* in a methanol-limited chemostat. Physiological responses due to the involvement of methanol oxidase as a key enzyme in methanol metabolism. Arch. Microbiol. **111**, 137–144 (1976)

De Duve, C., Baudhuin, P.: Peroxisomes (microbodies and related particles). Physiol. Rev. **46**, 323–356 (1966)

Eggeling, L., Sahm, H.: Derepression and partial insensitivity to carbon catabolite repression of the methanol dissimilatory enzymes in *Hansenula polymorpha*. Eur. J. Appl. Microbiol. Biotechnol. **5**, 197–202 (1978)

Egli, Th., van Dijken, J. P., Veenhuis, M., Harder, W., Fiechter, A.: Methanol metabolism in yeasts: regulation of the synthesis of catabolic enzymes. Arch. Microbiol. **124**, 115–121 (1980)

Feldman, J. M., Roche, J. M., Blum, J. J.: Monoamine oxidase and catechol-o-methyl transferase activity in *Tetrahymena*. J. Protozool. **24**, 459–462 (1977)

Kitamura, K., Kaneda, T., Yamamoto, Y.: Lysis of viable cells by enzymes of *Arthrobacter luteus*. Arch. Biochem. Biophys. **145**, 402–404 (1971)

Lang, E., Lang, H.: Spezifische Farbreaktion zum direkten Nachweis der Ameisensäure. Z. Anal. Chem. **260**, 8–10 (1972)

Lloyd, D.: The mitochondria of microorganisms. London, New York, San Francisco: Acad. Press 1974

Moor, H.: Die Gefrier-Fixation lebender Zellen und ihre Anwendung in der Elektronenmikroskopie. Z. Zell.-Forsch. **62**, 546–580 (1964)

Osumi, M., Imaizumi, F., Imai, M., Sato, H., Yamaguchi, H.: Isolation and characterization of microbodies from *Candida tropicalis* PK 233 cells grown on normal alkanes. J. Gen. Appl. Microbiol. **21**, 375–387 (1975)

La Rue, T. A., Spencer, J. F. T.: Utilization of organic nitrogen compounds by yeasts of the genus *Saccharomyces*. Antonie van Leeuwenhoek J. Microbiol. Serol. **34**, 153–158 (1968)

Roels, F., Wisse, E., de Prest, B., van der Meulen, J.: Cytochemical discrimination between catalases and peroxidases using diamino benzidine. Histochem. **41**, 281–312 (1975)

Roon, R. J., Even, L. E., Dunlop, P.: Methylamine and ammonium transport in *Saccharomyces cerevisiae*. J. Bacteriol. **122**, 502–509 (1975)

Sahm, H.: Metabolism of methanol by yeasts. Adv. Biochem. Engin. **6**, 77–103 (1977)

Tani, Y., Kato, N., Yamada, H.: Utilization of methanol by yeast. Adv. Appl. Microbiol. **24**, 165–186 (1978)

Veenhuis, M., van Dijken, J. P., Harder, W.: Cytochemical studies on the localization of methanol oxidase and other oxidases in peroxisomes of methanol-grown *Hansenula polymorpha*. Arch. Microbiol. **111**, 123–135 (1976)

Veenhuis, M., van Dijken, J. P., Pilon, S. A. F., Harder, W.: Development of crystalline peroxisomes in methanol-grown cells of the yeast *Hansenula polymorpha* and its relation to environmental conditions. Arch. Microbiol. **117**, 153–163 (1978)

Veenhuis, M., Keizer, I., Harder, W.: Characterization of peroxisomes in glucose-grown *Hansenula polymorpha* and their development after the transfer to cells into methanol-containing media. Arch. Microbiol. **120**, 167–175 (1979)

Van der Walt, J. P.: Utilization of ethylamine by yeasts. Antonie van Leeuwenhoek J. Microbiol. Serol. **28**, 91–96 (1962)

Weibel, E. R., Bolender, P.: Stereological techniques for electron microscopic morphometry. In: Principles and techniques of electron microscopy (M. A. Hayat, ed.). London: Van Nostrand Reinhold 1976

Yamada, H., Adachi, O., Ogata, K.: Amine oxidases of microorganisms. Part I. Formation of amine oxidase by fungi. Agric. Biol. Chem. **29**, 117–123 (1965a)

Yamada, H., Adachi, O., Ogata, K.: Amine oxidases of microorganisms. Part II. Purification and crystallization of amine oxidase of *Aspergillus niger*. Agric. Biol. Chem. **29**, 649–654 (1965b)

Yamada, H., Adachi, O., Ogata, K.: Amine oxidases of microorganisms. Part III. Properties of amine oxidase of *Aspergillus niger*. Agric. Biol. Chem. **29**, 864–869 (1965c)

Yamada, H., Adachi, O., Ogata, K.: Amine oxidases of microorganisms. Part IV. Further properties of amine oxidase of *Aspergillus niger*. Agric. Biol. Chem. **29**, 912–917 (1965d)

Yamada, H., Kumagai, H., Uwajima, T., Ogata, K.: Trimethylamine metabolism. I. Monomethylamine oxidase. Mem. Res. Inst. Food Sci. **27**, 1–13 (1966)

Yamada, H., Kishimoto, N., Kumagai, H.: Metabolism of N-substituted amines by yeasts. J. Ferment. Technol. **54**, 726–737 (1976)

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