

# Subcellular localization of vanillyl-alcohol oxidase in *Penicillium simplicissimum*

Marco W. Fraaije<sup>a</sup>, Klaas A. Sjollema<sup>b</sup>, Marten Veenhuis<sup>b</sup>, Willem J.H. van Berkel<sup>a,\*</sup>

<sup>a</sup>Department of Biomolecular Sciences, Laboratory of Biochemistry, Wageningen Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

<sup>b</sup>Laboratory for Electron Microscopy, Biological Centre, University of Groningen, Kercklaan 30, 9750 AA Haren, The Netherlands

Received 18 November 1997; revised version received 20 December 1997

**Abstract** Growth of *Penicillium simplicissimum* on anisyl alcohol, veratryl alcohol or 4-(methoxymethyl)phenol, is associated with the synthesis of relatively large amounts of the hydrogen peroxide producing flavoprotein vanillyl-alcohol oxidase (VAO). Immunocytochemistry revealed that the enzyme has a dual location namely in peroxisomes and in the cytosol. The C-terminus of the primary structure of VAO displays a WKL-COOH sequence which might function as a peroxisomal targeting signal type 1 (PTS1). As VAO activity results in production of hydrogen peroxide also the subcellular location of a recently characterized co-inducible catalase-peroxidase was studied. As VAO, this hydroperoxidase is also distributed throughout the cytosol and peroxisomes.

© 1998 Federation of European Biochemical Societies.

**Key words:** Vanillyl-alcohol oxidase; Covalent flavoprotein; Catalase-peroxidase; Peroxisome; *Penicillium simplicissimum*; Immunocytochemistry

## 1. Introduction

In 1992, a novel flavoprotein, vanillyl-alcohol oxidase (VAO), was isolated from the fungus *Penicillium simplicissimum* CBS 170.90 which can oxidize a wide variety of phenolic compounds [1,2]. The enzyme is specifically induced when the fungus is grown on veratryl alcohol, anisyl alcohol or 4-(methoxymethyl)phenol [3]. We have recently demonstrated that 4-(methoxymethyl)phenol represents a 'natural' inducer as VAO catalyzes the first step in the degradation of this phenolic methylether. However, with veratryl alcohol and anisyl alcohol, induction appears to be redundant for growth although the enzyme is produced in high quantities.

VAO is a homo-octameric covalent flavoprotein with each subunit harboring a histidyl bound FAD [1]. Recently, we have solved the crystal structure of VAO confirming the covalent bond between His<sup>422</sup> and the FAD cofactor [4]. Covalently bound flavin cofactors have been found in about 25 other enzymes while several hundred flavin dependent enzymes are known [5]. At present, the rationale for covalent flavinylation is still unclear. In the bacterial trimethylamine dehydrogenase, containing a cysteinyl bound FMN, the covalent linkage seems to prevent chemical modification of the flavin leading to inactivation [6]. For *p*-cresol methylhydroxylase, it was suggested that the linkage of FAD to a tyrosine residue results in a more efficient electron flow from the reduced flavin to the cytochrome subunit [7]. However, it has also been suggested that covalent flavinylation may improve

the in vivo stability of the enzyme or that it may be beneficial for the organism in times of decreased levels of available flavin [5].

Until now, eight eukaryotic covalent flavoproteins have been localized. It was found that all these enzymes are located in distinct cell organelles: monoamine oxidase, succinate dehydrogenase, dimethylglycine dehydrogenase, sarcosine dehydrogenase [8] and D-arabinono-1,4-lactone oxidase [9] are contained in mitochondria, L-gulonon- $\gamma$ -lactone oxidase is located in the endoplasmic reticulum [10], L-pipecolic acid oxidase and sarcosine oxidase in peroxisomes [11,12], and the plant reticuline oxidoreductase in vesicles [13]. For rat dimethylglycine dehydrogenase and yeast succinate dehydrogenase it was reported that covalent attachment of the FAD cofactor is stimulated when the precursor protein is imported and proteolytically processed in the mitochondria [8,14]. However, covalent flavinylation of these enzymes can also occur in the cytoplasm and holoenzyme can be imported in the mitochondria as well.

The present work describes an immunocytochemical study to determine the intracellular distribution of VAO in mycelium of *P. simplicissimum* CBS 170.90. Recently, we also purified a catalase-peroxidase from this fungus which represents the first characterized dimeric catalase-peroxidase of eukaryotic origin [15]. As VAO activity generates hydrogen peroxide as side-product and induction of VAO coincides with an elevated synthesis of this catalase-peroxidase, the induction and subcellular localization of catalase-peroxidase activity were studied as well.

## 2. Materials and methods

### 2.1. Microorganism and cultivation

All experiments were performed with *P. simplicissimum* CBS 170.90. Cells were grown as described earlier [3]. For immunocytochemical experiments, cells were harvested during the logarithmic growth phase (48 h after inoculation when grown on anisyl alcohol and 24 h after inoculation when grown on glucose).

### 2.2. Enzyme assays

The vanillyl-alcohol oxidase activity was assayed at 30°C and pH 10.0 with vanillyl alcohol as the aromatic substrate [1]. Catalase and peroxidase activities were assayed as described previously [15]. For peroxidase activity, 2,6-dimethoxyphenol was used as the electron donor. Glucose-6-phosphate dehydrogenase was assayed by the method of Bruinenberg et al. [16]. L-Glutamate dehydrogenase was determined by measuring the decrease of absorbance at 340 nm after adding 50- $\mu$ l aliquots of enzyme to 950  $\mu$ l 20 mM  $\alpha$ -ketoglutarate, 0.5 mM NADPH, 100 mM HEPES, pH 7.5.

### 2.3. Cell fractionation

Several methods were tested for the isolation of protoplasts. Invariably, with all tested lysing enzymes the efficiency of protoplast formation was very low, presumably due to a very rigid cell wall. There-

\*Corresponding author. Fax: +31 (317) 484801.  
E-mail: Willem.vanBerkel@FAD.BC.WAU.NL

fore, lysis of protoplasts by osmotic shock was not feasible. Grinding cells with quartz-sand, as described for *Neurospora crassa* [17], did neither result in efficient cell disruption. Finally, cells (10 g) were disrupted under liquid nitrogen by grinding frozen mycelia. For thawing, 75 ml 5 mM MES, pH 6.0, 1.0 M sorbitol was added. After filtration with a cheese cloth, the solution was centrifuged at  $16\,000\times g$  for 5 min at 4°C. The pellet was carefully resuspended in 5 mM MES, pH 6.0, 1.0 M sorbitol and applied to a 25–60% sucrose gradient in 5 mM MES, pH 6.0. Gradients were centrifuged (100 min, 32 000 rpm) in a swinging bucket rotor (TST 4114 rotor, Centrikon T-1055 centrifuge).

#### 2.4. Polyclonal antibodies

Rabbit polyclonal antibodies against VAO and catalase-peroxidase were obtained as described earlier [15]. The antisera were purified by incubation with glucose-grown mycelia to remove aspecific cell wall binding components. Subsequently, the antisera were further purified by hydrophobic interaction chromatography using a phenyl-Sepharose column (elution gradient: 0.6–0 M ammonium sulfate in 20 mM potassium phosphate, pH 7.5).

#### 2.5. Immunocytochemical experiments

Immunocytochemistry was performed on ultrathin sections of Unicryl (British Biocell International) embedded cells, using purified polyclonal antibodies and goat anti-rabbit IgG conjugated to gold (Amersham, UK), basically according to the method of Slot and Geuze [18].

### 3. Results

#### 3.1. Enzyme induction

Earlier studies have shown that VAO activity is induced when *P. simplicissimum* is grown on some specific aromatic compounds [3]. It was also observed that, during growth on veratryl alcohol, relatively high amounts of a dimeric catalase-peroxidase were produced in parallel with production of an atypical catalase [15]. As VAO activity results in the generation of hydrogen peroxide as a side-product, these enzymes may represent the response of the organism to eliminate this toxic compound. Table 1 shows that on all tested growth substrates the atypical catalase is produced at a rather constant level, while the catalase-peroxidase is present at a higher level when the fungus is grown on aromatic compounds. The induction of catalase-peroxidase is highest during growth on the VAO-inducing growth substrates. However, from the data of Table 1, it is clear that the synthesis of catalase-peroxidase activity is not as strictly regulated as that of VAO.

#### 3.2. Cell fractionation studies

Fig. 1 shows the distribution of various *P. simplicissimum* enzyme activities after sucrose density centrifugation of an organellar pellet prepared from cells grown on veratryl alcohol. Activity of the mitochondrial marker enzyme L-glutamate

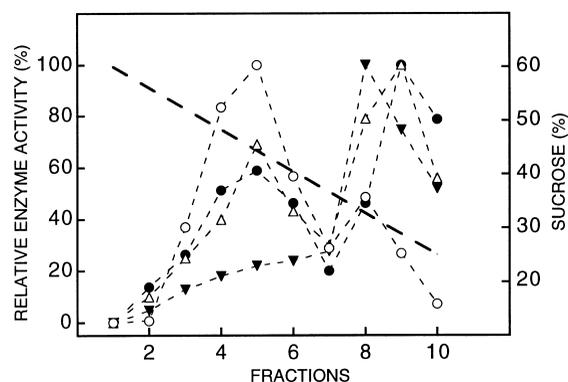


Fig. 1. Distribution of VAO ( $\Delta$ ), peroxidase ( $\circ$ ), catalase ( $\bullet$ ) and L-glutamate dehydrogenase ( $\blacktriangledown$ ) activity in a 25–60% (w/v) sucrose gradient (---) after sucrose density centrifugation.

dehydrogenase was primarily found in protein fractions with a density of  $1.15\text{ g/cm}^3$ . The three other enzyme activities tested showed a bimodal distribution. The relative high level of activity in the  $1.21\text{--}1.24\text{ g/cm}^3$  density fractions indicated that these enzymes are compartmentalized. Activity of these enzymes observed in the lower density fractions may originate from organelle leakage, possibly due to the relatively harsh method for cell breakage. A similar bimodal distribution of catalase activity has been observed before [19,20]. The relatively high catalase activity in the lower density fractions of the gradient, as compared to the peroxidase activity in these fractions, may in part also result from some residual atypical catalase activity.

#### 3.3. Immunocytochemical localization

Western blot analysis has shown that the antisera raised against VAO and catalase-peroxidase are specific for the corresponding proteins [3]. By using the purified polyclonal antibodies, the subcellular localization of both VAO and catalase-peroxidase was determined (see Section 2). Fig. 2A shows the labeling pattern on ultrathin sections of anisyl alcohol-grown cells, using purified  $\alpha$ -VAO antibodies. It is evident that the labeling is not restricted to a specific cell compartment, but localized on the peroxisomal matrix and the cytosol as well, including the nuclei. Fig. 2A also shows that significant label is absent on other cell compartments, including the mitochondria. In accordance with the absence of VAO activity, no significant labeling was observed in glucose-grown control cells (Fig. 2B). Using antibodies raised against catalase-peroxidase, an identical subcellular labeling pattern was observed: predominantly peroxisomes and cytosol were labeled on both glucose- and anisyl alcohol-grown cells (Fig. 3). These

Table 1  
Growth substrate dependent induction of VAO, catalase and peroxidase activity in *P. simplicissimum*

Growth substrate	VAO <sup>a</sup>	Atypical catalase <sup>b</sup>	Catalase-peroxidase <sup>c</sup>
Anisyl alcohol	74.5	36.5	55.9
Veratryl alcohol	69.8	38.7	56.6
Vanillyl alcohol	< 1	27.1	31.8
Vanillic acid	< 1	50.4	31.9
Homovanillyl alcohol	< 1	48.3	33.8
Glucose	< 1	25.4	5.0

<sup>a</sup>VAO activity in mU/mg.

<sup>b</sup>Atypical catalase activity in U/mg (as measured at pH 8.5).

<sup>c</sup>Catalase activity of catalase-peroxidase in U/mg (= catalase activity measured at pH 6.4 corrected for activity of atypical catalase activity at pH 8.5 (see [15])).

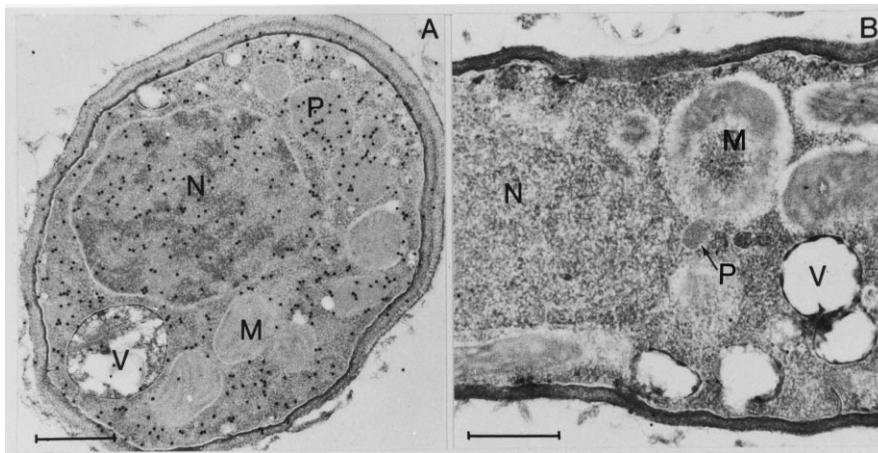


Fig. 2. Immunogold labeling of VAO in anisyl alcohol- (A) and glucose-grown (B) *P. simplicissimum* cells (P, peroxisome; M, mitochondrion; N, nucleus; V, vacuole; bar represents 0.5  $\mu\text{m}$ ).

results are in line with the fact that glucose does not induce VAO production whereas catalase-peroxidase is expressed under all growth conditions tested (Table 1).

#### 4. Discussion

In this study, we have shown that the covalent flavoprotein VAO has a bimodal distribution and is located in peroxisomes and the cytosol of *P. simplicissimum*. The peroxisomal localization of VAO was not unexpected as it is known that various hydrogen peroxide-producing flavoenzymes, e.g. sarcosine oxidase [12], D-amino acid oxidase [21], alcohol oxidase [22] and acyl-CoA oxidase [23] are compartmentalized in these organelles. Moreover, being a substrate inducible enzyme, VAO shows several features which are typical for many peroxisomal matrix enzymes. As a significant amount of VAO was localized in peroxisomes, the primary structure of VAO [24] was analyzed for the presence of a peroxisomal targeting signal (PTS). It was found that at the extreme C-terminus a WKL-COOH tripeptide sequence is present which resembles the well-known PTS1, characterized by the SKL-COOH consensus sequence [25]. Most of the reported fungal flavin-dependent oxidases contain a PTS1 [21,26–31] although for acyl-CoA oxidases other targeting mechanisms may be operative [23]. Therefore, it is likely that the VAO C-terminal sequence represents a novel PTS1 variant. As only part of the protein is located in peroxisomes, translocation may not be fully efficient due to a limited recognition of this PTS1 variant. As an alternative, the partial translocation of VAO into peroxisomes may be caused by a relatively fast rate of VAO syn-

thesis exceeding the import capacity. Another striking observation with respect to the subcellular location of VAO is the fact that a large portion of VAO is present in the nuclei. The presence of hydrogen-peroxide generating activity in nuclei is remarkable as this could lead to deleterious effects. However, peroxisomal matrix enzymes including  $\text{H}_2\text{O}_2$ -producing enzymes also accumulate in nuclei of per mutants of methylotrophic yeast [32] and it is still not known how the cell deals with this potentially hazardous event. In line with this, the presence of VAO in the cytosol may indicate that the translocation of VAO within the cell is not optimally regulated. A rationale behind this could be that the VAO gene is a result of a relatively recent gene transfer. The plasmid-encoded flavocytochrome *p*-cresol methylhydroxylase isolated from *Pseudomonas putida* is a possible candidate for such an event as this enzyme is highly homologous to VAO: both the primary sequence and 3-D structure are very similar to VAO [4,7].

Generally, hydrogen peroxide produced in peroxisomes is efficiently eliminated by the action of peroxisomal catalase activity. However, hydrogen peroxide producing and degrading enzymes have also been detected in other cell compartments. For example, the mammalian monoamine oxidase is localized in mitochondria and in several fungi, flavin-dependent oxidases are excreted. *Aspergillus niger* produces large amounts of the extracellular glucose oxidase resulting in acidification of the medium [33]. Other organisms, like the fungus *Talaromyces flavus* and the red alga *Chondrus crispus*, produce extracellular sugar oxidases as a defense system generating extracellular toxic hydrogen peroxide [34,35]. For white-rot fungi, the extracellular production of hydrogen peroxide by

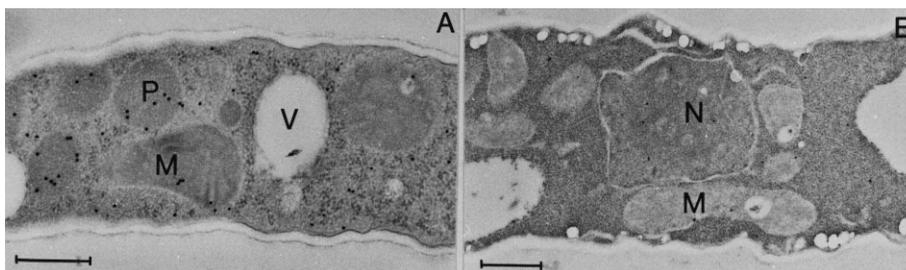


Fig. 3. Immunogold labeling of catalase-peroxidase in anisyl alcohol- (A) and glucose-grown (B) *P. simplicissimum* cells (P, peroxisome; M, mitochondrion; N, nucleus; V, vacuole; bar represents 0.5  $\mu\text{m}$ ).

action of flavin-dependent oxidases is crucial to sustain the enzymatic lignin degradation mechanism [36]. Previously, we have shown that *P. simplicissimum* produces two hydroperoxidases: an atypical periplasmic catalase and an intracellular catalase-peroxidase [15]. By cell fractionation and immunolocalization we have established that the catalase-peroxidase is located throughout the cell and thereby may decompose the hydrogen peroxide produced by VAO. An increase in production of catalase-peroxidase concurrent with VAO induction is in keeping with this hypothesis. It has been shown that, due to a low affinity for hydrogen peroxide, catalases only function in the degradation of hydrogen peroxide when it is present at the site of its generation, e.g. in peroxisomes. However, for catalase-peroxidases the physiological electron donor is not yet known and, consequently, its efficiency for peroxidase reactions is also unknown. Cytochrome *c* peroxidase plays an essential role in the intracellular degradation of hydrogen peroxide in yeasts [37]. As catalase-peroxidases show sequence homology with cytochrome *c* peroxidase [38], these hydroperoxidases may be effective alternatives in decomposing subcellularly produced hydrogen peroxide.

VAO represents the first example of a covalent flavoprotein which is not strictly compartmentalized. The presence of active octameric VAO in both peroxisomes and cytosol demonstrates that for this enzyme no specific organelle-bound assembly factors are required for flavinylation and oligomerization/activation. This is in contrast with the FAD-containing alcohol oxidases from methylotrophic yeasts which in WT cells only assemble into octamers after import of FAD-lacking monomers in the peroxisomes and subsequent FAD binding [39]. The self-assembly and autoflavinylation of VAO is in keeping with the production of active enzyme in heterologous hosts like *A. niger* and *Escherichia coli* [24].

**Acknowledgements:** We thank Jan Schel (Department of Plant Cytology and Morphology, WAU) for his support and interest.

## References

- [1] De Jong, E., Van Berkel, W.J.H., Van Der Zwan, R.P. and De Bont, J.A.M. (1992) *Eur. J. Biochem.* 208, 651–657.
- [2] Fraaije, M.W., Veeger, C. and Van Berkel, W.J.H. (1995) *Eur. J. Biochem.* 234, 271–277.
- [3] Fraaije, M.W., Pikkemaat, M. and van Berkel, W.J.H. (1997) *Appl. Environ. Microbiol.* 63, 435–439.
- [4] Mattevi, A., Fraaije, M.W., Mozzarelli, A., Olivi, L., Coda, A. and van Berkel, W.J.H. (1997) *Structure* 5, 907–920.
- [5] Decker, K.F. (1993) *Annu. Rev. Nutr.* 13, 17–41.
- [6] Mewies, M., Basran, J., Packman, L.C., Hille, R. and Scrutton, N.S. (1997) *Biochemistry* 36, 7162–7168.
- [7] Kim, J., Fuller, J.H., Kuusk, V., Cunane, L., Chen, Z., Mathews, F.S. and McIntire, W.S. (1995) *J. Biol. Chem.* 270, 31202–31209.
- [8] Otto, A., Stolz, M., Sailer, H. and Brandsch, R. (1996) *J. Biol. Chem.* 271, 9823–9829.
- [9] Huh, W.K., Kim, S.T., Yang, K.S., Seok, Y.J., Hah, Y.C. and Kang, S.O. (1994) *Eur. J. Biochem.* 225, 1073–1079.
- [10] Yagi, K. and Nishikimi, M. (1994) In: *Flavins and Flavoproteins 1993* (Yagi, K., Ed.) pp. 799–802, W. de Gruyter, Berlin.
- [11] Mihalic, S.J. and McGuinness, M.C. (1991) In: *Flavins and Flavoproteins 1990* (Curti, B., Ronchi, S. and Zanetti, G., Eds.) pp. 881–884, W. de Gruyter, Berlin.
- [12] Reuber, B.E., Karl, C., Reimann, S.A., Mihalik, S.J. and Dodt, G. (1997) *J. Biol. Chem.* 272, 6766–6776.
- [13] Kutchan, T.M. and Dittrich, H. (1995) *J. Biol. Chem.* 270, 24475–24481.
- [14] Robinson, K.M. and Lemire, B.D. (1996) *J. Biol. Chem.* 271, 4055–4060.
- [15] Fraaije, M.W., Roubroeks, H.P., Hagen, W.R. and van Berkel, W.J.H. (1996) *Eur. J. Biochem.* 235, 192–198.
- [16] Bruinenberg, P.M., van Dijken, J.P. and Scheffers, W.A. (1983) *J. Gen. Microbiol.* 129, 965–971.
- [17] Kionka, C. and Kunau, W.H. (1985) *J. Bacteriol.* 161, 153–157.
- [18] Slot, J.W. and Geuze, H.J. (1984) In: *Immunolabeling for Electron Microscopy* (Polack, J.M. and Varndell, I.M., Eds.) pp. 129–142, Elsevier, Amsterdam.
- [19] Goodman, J.M. (1985) *J. Biol. Chem.* 260, 7108–7113.
- [20] Kamasawa, N., Ohtsuka, I., Kamada, Y., Ueda, M., Tanaka, A. and Osumi, M. (1996) *Cell. Struct. Funct.* 21, 117–122.
- [21] Pilone Simonetta, M., Perotti, M.E. and Pollegioni, L. (1991) In: *Flavins and Flavoproteins 1990* (Curti, B., Ronchi, S. and Zanetti, G., Eds.) pp. 167–170, W. de Gruyter, Berlin.
- [22] Veenhuis, M., van Dijken, J.P., Pilon, S.A.F. and Harder, W. (1978) *Arch. Microbiol.* 117, 153–163.
- [23] Small, G.M., Szabo, L.J. and Lazarow, P.B. (1988) *EMBO J.* 7, 1167–1173.
- [24] Benen, J.A.E., Wagemaker, M.J.M., Sánchez-Torres, P., Fraaije, M.W., van Berkel, W.J.H. and Visser, J. (1997) in preparation.
- [25] Gould, S.J., Keller, G.A., Hosken, N., Wilkinson, F. and Subramani, S. (1989) *J. Cell Biol.* 108, 1657–1664.
- [26] Yoshida, N., Sakai, Y., Isogai, A., Fukuya, H., Yagi, M., Tani, Y. and Kato, N. (1996) *Eur. J. Biochem.* 242, 499–505.
- [27] Takahashi, M., Pischetsrieder, M. and Monnier, V.M. (1997) *J. Biol. Chem.* 272, 12505–12507.
- [28] Isogai, T., Ono, H., Ishitani, Y., Kojo, H., Ueda, Y. and Kohsaka, M. (1990) *J. Biochem. (Tokyo)* 108, 1063–1069.
- [29] Faotto, L., Pollegioni, L., Cecilian, F., Ronchi, S. and Pilone, M.S. (1995) *Biotech. Lett.* 17, 193–198.
- [30] Schilling, B. and Lerch, K. (1995) *Mol. Gen. Genet.* 247, 430–438.
- [31] Hansen, H., Didion, T., Thiemann, A., Veenhuis, M. and Roggenkamp, R. (1992) *Mol. Gen. Genet.* 235, 269–278.
- [32] Van Der Klei, I.J., Hilbrands, R.E., Swaving, G.J., Waterham, H.R., Vrieling, E.G., Titorenko, V.I., Cregg, J.M., Harder, W. and Veenhuis, M. (1995) *J. Biol. Chem.* 270, 17229–17236.
- [33] Witteveen, C.F.B., Veenhuis, M. and Visser, J. (1992) *Appl. Environ. Microbiol.* 58, 1190–1194.
- [34] Sullivan, J.D. and Ikawa, M. (1973) *Biochim. Biophys. Acta* 309, 11–20.
- [35] Stosz, S.K., Fravel, D.R. and Roberts, D.P. (1996) *Appl. Environ. Microbiol.* 62, 3183–3186.
- [36] Ander, P. and Marzullo, L. (1997) *J. Biotechnol.* 53, 115–131.
- [37] Verduyn, C., Giuseppin, M.L.F., Scheffers, W.A. and van Dijken, J.P. (1988) *Appl. Environ. Microbiol.* 54, 2086–2090.
- [38] Welinder, K.G. (1991) *Biochim. Biophys. Acta* 1080, 215–220.
- [39] Evers, M.E., Titorenko, V., Harder, W., van der Klei, I. and Veenhuis, M. (1996) *Yeast* 12, 917–923.