Methylotrophic yeasts possess a respiratory type of metabolism and during growth an accumulation of potentially cytotoxic species (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) takes place (13, 27). The catalase [E.C.1.11.1.6] and superoxide dismutase [E.C.1.15.1.1] enzymes play a key role in cellular defense against the reactive species (9).

The intracellular localization of catalase enzyme has been under debate for many years. It is a topic widely investigated and the initial localization in peroxisomes has been determined by a series of cytochemical and biochemical studies which also describe its position in other cellular compartments. This approach has been used by number of authors (8, 18, 20, 32). Michailova et al. (20) have isolated a pure heavy mitochondrial fraction from Candida boidinii and have provided evidences for catalase activity.

At present it is well documented with Saccharomyces cerevisiae yeast cells that mitochondria possess catalase A enzyme (24). It has been shown that catalase A, although primary considered as a peroxisomal protein, could also be independently target to mitochondria (25).

Given this data, it is important to perform broader investigations on catalase enzyme in methylotrophic yeasts. In the present work, we have studied the mitochondrial localization of catalase using three different strains methylotrophic yeasts: Pichia pastoris, Pichia pini and Hansenula polymorpha and demonstrated that similarly to S. cerevisiae yeast, methylotrophic ones possess constitutive transport of catalase into both organelles.

Materials and Methods

Microorganisms and growth conditions

The yeasts used in this investigation were, as follows: Pichia pastoris X-33 (Invitrogen), Hansenula polymorpha CBS 4732 and Pichia pini NBIMCC 8360. The strains were cultivated in liquid YP medium (1% Yeast Extract, 1% Bacto-Peptone) supplemented either with 2 % glucose (YPD), 1 % methanol (YPM) or 1 % glycerol (YPG) at 30°C on a reciprocal shaker (204 rpm).

Cell-free extract preparation

Cells from 6, 12, 20, 30, 48 and 72 h of cultivation were harvested by centrifugation at 800 x g for 10 minutes and washed twice with distilled H$_2$O. Cell wall disruption was carried out by spheroplasting according to the procedure of Defontaine et al. (3). The cell debris was removed by centrifugation at 1000 g and 4°C for 10 min. The supernatant containing the crude yeast cell organelles was again centrifuged at 25 000 g and 4°C for 10 min. The supernatants obtained were used for enzymatic analyses.

Subcellular fractionation and Nycodenz gradients

For cell fractionation, yeast cells were grown 20 h to reach end of logarithmic phase. Spheroplasts were generated by the procedure of Defontaine et al. (3) and unlysed cells, nuclei and cell debris were removed by centrifugation at 1000 g and 4°C for 10 min. The supernatant containing the crude yeast cell organelles was again centrifuged at 25 000 g and 4°C for 20 min, and the crude organelle fraction was resuspended in a total volume of 1 ml of ice-cold buffer (28).
volume of 5 mM Mes/KOH buffer (pH 6.0) containing 0.24 M sucrose (to provide osmotic protection for the peroxisomes) and 1 mM EDTA. Purity of such an organelle fraction was routinely determined by Western blot analysis and activity assays of marker enzymes, such as glucose-6-phosphate dehydrogenase, hexokinase, succinate dehydrogenase, and isocitrate lyase, indicating that the organelle fraction was highly enriched for yeast mitochondria and peroxisomes (results not shown). For the separation of cell organelles, in particular for separating peroxisomes from mitochondria, the crude organelle fraction was layered on top of a Nycodenz (ICN) step gradient consisting of 17%, 25% and 35% Nycodenz in the same buffer, and 4°C for 90 min in a SW50Ti rotor (Beckman) and the gradient was subsequently fractionated from the bottom. Each fraction (1 ml) was diluted 5-fold with Mes/KOH buffer (5 mM, pH 6.0, containing 0.24 M sucrose and 1 mM EDTA), and the Nycodenz was removed by a final centrifugation step at 15 000 g for 20 min. For enzyme assays, gradient purified organelles were ruptured by the addition of Triton X-100 to a final concentration of 1%. Yeast mitoplasts were isolated from Nycodenz-purified mitochondria by hypotonic swelling-shrinking treatment according to Zinser and Daum (37).

Biochemical analyses
Catalase [EC: 1.11.1.6] activity was determined spectrophotometrically according to Aebi, (1) and Upadhya et al. (31).

D-amino acid oxidase [EC: 1.4.3.3] activity was measured, as described by Lichtenberg and Wellner (17).

Fumarase [EC: 4.2.1.2] activity was assayed according to the procedure of Kanarek and Hill (10).

Analysis of carbon source utilization
Glucose was determined by the method of Somogy (29) and Nelson (23). Methanol was determined by the method of Gonzalez-Rodriguez et al. (7). Glycerol was determined according to the procedure described by Moller and Roomi (22).

Cell dry weight estimation
Cell dry weight was determined gravimetrically after drying washed cells to constant weight at 105°C.

Determination of kinetic parameters
Specific growth rate (μ) and maximal cell yield (Ys) were calculated according to Monod kinetics as described by Pirt (26).

Protein determination
Protein content was determined by the method of Lowry et al. (19). Bovine serum albumin (Sigma St. Louis, MO, USA) was used as a standard.

Electron microscopy
Electron microscopy was performed as described previously in Michailova et al. (20). Whole yeast cells were field with 1.5 % potassium permanganate for 20 min at room temperature washed and stained with 1% (w/v) uranyl acetate for 1 h. After dehydratation in graded ethanol series the material was embedded in Epon 812 resins. The ultrathin sections of the cells were obtained on an Ultracut Reichert apparatus and were stained with uranyl acetate and lead citrate. The observations were carried out on a Zeiss EM 10C electron microscope at 60 or 80 kV.

Results and Discussion
Influence of carbon source on growth, proliferation of mitochondria and peroxisomes, and catalase activity
The catalase enzyme activity was investigated during batchwise cultivation of three methylotrophic yeast strains (Pichia pastoris X-33, Hansenula polymorpha CBS 4732 and Pichia pini NBIMCC 8360) on standard YPD medium. Cultivation of the cells was performed for 72 h and samples were withdrawn during different growth phases of the culture in order to determine cell dry weight and glucose consumption (Fig. 1a). It is evident that a diauxic growth takes place as glucose is exhausted at the 20th hour of cultivation and cells enter stationary phase at 48th h. The specific growth rate and maximal cell yield were calculated according to Monod Equation (Table 1). Obtained results for μmax = 0.30 ± 0.32 h⁻¹ and Ymax = 0.27 ± 0.30 during growth on glucose are in accordance with the data published from Sola et al. (28) for metabolic state of some methylotrophic yeasts. Thus the chosen strains for investigations of compartmentalization of catalase enzyme could be considered as effectively growing yeasts.

![TABLE 1](attachment:image)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ys/glucose</th>
<th>Ys/glycerol</th>
<th>Ys/methanol</th>
<th>μ/glucose</th>
<th>μ/glycerol</th>
<th>μ/methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pichia pastoris X-33</td>
<td>0.30 ± 0.05</td>
<td>0.32 ± 0.05</td>
<td>0.49 ± 0.06</td>
<td>0.32 ± 0.015</td>
<td>0.149 ± 0.023</td>
<td>0.101 ± 0.12</td>
</tr>
<tr>
<td>Pichia pini NBIMCC 8360</td>
<td>0.28 ± 0.01</td>
<td>0.30 ± 0.03</td>
<td>0.48 ± 0.09</td>
<td>0.31 ± 0.011</td>
<td>0.150 ± 0.025</td>
<td>0.102 ± 0.06</td>
</tr>
<tr>
<td>Hansenula polymorpha CBS 4732</td>
<td>0.27 ± 0.02</td>
<td>0.30 ± 0.05</td>
<td>0.47 ± 0.01</td>
<td>0.30 ± 0.023</td>
<td>0.152 ± 0.22</td>
<td>0.104 ± 0.09</td>
</tr>
</tbody>
</table>
Further, samples of biomass were processed and crude enzyme extracts prepared. A spectrophotometrical analysis of the total catalase activity at different hours of growth is shown in Fig. 2. Due to the presence of glucose in the media at the 6th h of cultivation, no catalase activity was detected as it is subjected to glucose repression (14, 36). The highest catalase activity was measured after 48 h of cultivation, which coincided with the late exponential – early stationary growth phase of the cultures. This increase in catalase activity, after the exhaustion of glucose, is consistent with the available data for glucose repression in methylotrophic yeasts on alcohol oxidase and catalase synthesis (14). Additionally, the different amino compounds supplemented in YPD media are metabolized from yeast and other fungi through hydrogen producing oxidases (33). So, correlation of the catalase activity with the accumulation of its specific substrate (H₂O₂) obviously takes place.

In order to investigate the influence of different carbon sources on proliferation of cellular substructures corresponding to catalase localization, except glucose, both glycerol and methanol were used as growth substrates. It is known that the catabolic pathway of glycerol in yeasts involves passive diffusion across the membrane, phosphorylation by glycerol kinase in cytosol, and oxidation by mitochondrial glycerol phosphate ubiquinone oxireductase (5). During growth on glycerol, the yeast cells are highly enriched with mitochondria and respiration rate is enhanced to maximum (6). The utilization of methanol proceeds via ROS producing alcohol oxidase, located into peroxisomes, thus leading to active peroxisomal proliferation in yeast cells (15, 20).
**Hansenula polymorpha CBS4732**  
**Pichia pini NBIMCC 8360**

**Fig. 3.** Electron micrographs of *Hansenula polymorpha* CBS4732 and *Pichia pini* NBIMCC 8360. Bar = 0.2 μm.

The ultrathin sections were obtained after growth of tested methylotrophic yeast strains in rich media supplemented with different carbon sources: (a) glucose, (b) glycerol and (c) methanol.
From the growth curves shown on Fig. 1b and Fig. 1c we calculated specific growth rate on glycerol and methanol that were respectively $0.149 + 0.152$ h$^{-1}$ and $0.101 + 0.104$ h$^{-1}$. Obtained cell yield of glycerol substrate is $Y_{\text{Gly}} = 0.30 + 0.32$ and of methanol - $Y_{\text{Meth}} = 0.47 + 0.49$, which demonstrate the effective utilization of these substrates by all tested strains and confirms the active metabolic status for targeted structures under investigation.

Consequently obtained biomass, enriched in mitochondria and peroxisomes, was subjected to enzymatic lyses and a spectrophotometrical analysis of the total catalase activity was performed (Fig. 2). We found that catalase enzyme is present during the whole period of cultivation and its maximum is measured at 48th h of cell growth, which coincides with exponential phase. These data suggest full induction of catalase when a respiratory carbon sources are used, and support the results previously obtained in our laboratory for subsequent induction of catalase synthesis after glucose depletion (20, 24, 36). Further, comparative analysis between catalase activity in Hansenula polymorpha, Pichia pastoris and Pichia pini, cultured on the three different carbon sources, was performed. It was shown that maximal catalase synthesis appears when cells are grown on methanol, lower in glycerol and the lowest catalase activity was detected when glucose is used as sole carbon source (average $38.16 > 31.72 > 26.88$ U mg$^{-1}$, respectively).

The abundance of the cellular organelles (i.e. mitochondria and peroxisomes) has been visualized by performance of electron microscopy assay of the biomass of different samples (Fig. 3). The study of strains grown in glucose containing media revealed single small peroxisomal structures and few under differentiated mitochondria. In YPG medium yeast cells contained only few small mitochondria, slightly increase in size compared to the organelles present during growth of cells on glucose, as well as numerous elongated or enlarged mitochondria. However, in methanol-grown cells peroxisomal proliferation was strongly induced and decrease of the number of mitochondria was detected.

**Compartmentalization of catalase activity in methylotrophic yeast cells**

In our previous studies (24, 25) we have already demonstrated that peroxisomal catalase A of Saccharomyces cerevisiae could successfully be targeted both to peroxisomes and mitochondria. In order to explore catalase distribution in yeast cells from Pichia pastoris X-33, Pichia pini NBIMCC 8360 and Hansenula polymorpha CBS4732 strains, the already isolated cell free extracts were subjected to cell fractionation. The resulted subcellular fractions were purified by ultracentrifugation through a Nycodenz step gradient and subsequently analyzed for protein content, D-amino acid oxidase and fumarase activity. The results presented in Table 2 clearly indicate that there were two peaks in protein distribution: in fraction 4 and fraction 8. As the maximum of D-amino acid oxidase activity is located in fraction 4 and the maximum of fumarate activity was detected in fraction 8 it could be concluded that these fractions are highly enriched in peroxisomes and mitochondria, respectively. The observed sedimentation profile of catalase enzyme in Nycodenz fractions (Fig. 4) indicates that sole peroxisomal catalase enzyme detected in methylotrophic yeast species, resembling peroxisomal catalase A of Saccharomyces cerevisiae, probably also is efficiently co-targeted to two different organelles, peroxisomes and mitochondria.

**TABLE 2**

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>Enzyme activity</th>
<th>Protein concentration [mg ml$^{-1}$]</th>
<th>DAAO [U mg$^{-1}$]</th>
<th>Fumarase [U mg$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>0.25±0.02</td>
<td>0.05±0.02</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.41±0.03</td>
<td>0.23±0.04</td>
<td>0.15±0.02</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.50±0.12</td>
<td>0.23±0.04</td>
<td>0.13±0.03</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>1.43±0.05</td>
<td>3.63±0.02</td>
<td>0.32±0.04</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.32±0.05</td>
<td>0.82±0.11</td>
<td>0.3±0.02</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>0.25±0.02</td>
<td>0.32±0.08</td>
<td>0.31±0.06</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>1.38±0.02</td>
<td>0.22±0.04</td>
<td>0.6±0.06</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>2.51±0.11</td>
<td>0.25±0.03</td>
<td>1.2±0.09</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>0.92±0.01</td>
<td>0.28±0.06</td>
<td>0.8±0.08</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>0.91±0.04</td>
<td>0.12±0.02</td>
<td>0.3±0.04</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>0.68±0.03</td>
<td>0.08±0.01</td>
<td>0.15±0.02</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>0.19±0.01</td>
<td>0.04±0.01</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>0.15±0.01</td>
<td>0.02±0.01</td>
<td>0.2±0.02</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>0.17±0.02</td>
<td>0.5±0.04</td>
<td>0.5±0.05</td>
</tr>
</tbody>
</table>

Next, the already isolated cell fractions were studied for catalase activity in order to check its organelle distribution during yeast growth on glucose, glycerol and methanol. The obtained data (Fig. 4) indicated that during utilization of methanol, catalase enzyme is present in both organelles as the majority of activity was detected in peroxisomes. On the contrary, when cells were cultivated on the polyvalent-alcoholic carbon source glycerol, catalase enzyme was predominantly targeted to mitochondria. When cells were grown in YPD media catalase was relatively equal distributed between the two organelles. Evidently, when glycerol is consumed from yeast cells and active respiration is induced, catalase enzyme is strongly required in mitochondria. Here, we determine that about 65 % of catalase activity was localized in the mitochondria. The opposite phenomenon could be observed under peroxisomes-inducing conditions. When methanol is added to the nutrient media the majority of catalase protein (80 %) was located into peroxisomes. In all cases, catalase was constitutively targeted to both organelles and only the percentage of enzyme distribution differs depending on growth conditions.
It is reasonable to suggest that future investigation with this model system will provide not only clear vision for processes of oxidation in eukaryotic cell but will help for better understanding of mechanisms of cellular aging and apoptosis.

Acknowledgements
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REFERENCES

Fig 4. Activity and distribution of peroxisomal and mitochondrial catalase during cell growth on different carbon sources

Conclusions
The data obtained from our investigations, for presence of mitochondrial catalase activity in fermentative (Saccharomyces), as well as in respiratory (Pichia and Hansenula) type of yeasts, allow us to speculate that targeting of catalase enzyme to mitochondria is a common feature to all yeast microorganisms.

Along side our investigations, many different studies provided strong evidence for the key role of catalase and superoxide dismutase enzymes for cellular aging and senescence, as well as for development of different diseases as Alzheimer, cancer, rheumatoid arthritis, arteriosclerosis, and etc. (11, 34). It was shown also that targeting catalase to the mitochondria provides better protection than cytosolic expression against H2O2-induced injury (2, 30).

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