Chapter 7

Materials and methods

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7.1. Yeast strain and growth conditions

C. halophila CBS 4019, was maintained at 4°C in solid YEPD medium (2 % (w/v) glucose, 2 % (w/v) peptone and 1 % (w/v) yeast extract). Cells were cultivated in liquid mineral medium (MM), using 0.5 % (w/v) ammonium sulfate as nitrogen source [van Uden, 1967] and different carbon sources, D-glucose, D-mannitol, glycerol and ethanol (2 % (w/v)). Media were supplemented with several NaCl concentrations according to text. Growth was assayed in batch cultures, with a liquid/air ratio of 1:2 for glucose growing cells and 1:4 for the other carbon sources. Incubation was performed at 30 °C, with orbital shaking at 160 r.p.m. (Certomat HK, B. Braun, Germany). Growth was monitored measuring the optical density (O.D.), absorbance at 640 nm, in a Spectronic 21 (Baush & Lomb). Parallel dry weight determinations were performed taking 10 ml samples which were filtered through ME 25/41 ST mixed ester membranes (Schleicher & Schuell, Germany), washed with an identical volume of distilled water and dried at 80 °C overnight. The specific growth rates (μ_g) during the exponential growth were calculated using O.D. values. Yield coefficient ($Y_{X/S}$) was determined as the ratio between dry weight and the remaining substrate concentration in the stationary phase.

7.2. Preparation of intracellular soluble fractions

Cells grown in the presence and absence of sodium chloride were harvested in late exponential growth phase (O.D. 0.8 - 0.9) by centrifugation for 2 min at 9 000 r.p.m. using a 4K10 centrifuge (B. Braun, Germany), and washed twice with ice-cold distilled water. Cells were lysed with trichloroacetic acid (3 % w/v) during 30 min at 70°C. After digestion, cell debris were separated by centrifugation, for 30 min at 15 000 r.p.m. at 4 °C. The supernatant was collected and analyzed by HPLC as mentioned below.

7.3. Estimation of sugar and other compounds concentration

Sugars, organic acids and polyols intracellular concentration were determined by **H**igh **P**erformance **L**iquid **C**hromatography (HPLC) using the internal standard method as described by [Lages and Lucas, 1997]. When samples were obtained from media containing NaCl, enzymatic determinations were made instead. Glycerol, glucose, acetic acid and ethanol were thus determined using Boheringer Manheim kits, references 148270, MPR3124036, 148261 and 176290, respectively.

7.4. Determination of internal pH

The internal pH was determined based on the general method described by Rottenberg, (1979), which measures the relative distribution of [^{14}C] propionic acid. The method was followed as described by Pampulha and Loureiro-Dias (1989).

7.5. Determination of H^+-ATPase activity

Cells were harvested in late exponential growth phase (O.D. 0.8-0.9), washed twice and ressuspended in ice-cold distilled water with or without the same sodium chloride concentration as

growth media. Cell suspension final concentration was 25 to 30 (mg dry weight) ml^{-1}. Assays were performed incubating 0.5 ml cell suspension with 4.5 ml distilled water (with or without salt, so to achieve the same concentration used during growth), for 2 to 3 min at 30 °C, pH 5.0 with gentle magnetic stirring. pH was adjusted using HCL 10 mM or NaOH 10 mM when necessary. Assays were performed using a pulse of 100 mM glucose [Serrano, 1980]. The glucose-induced acidification was measured using a pH electrode (G-202 B, Radiometer Copenhagen) connected to a pH Meter (Radiometer Copenhagen). The curve was recorded in a flat-bed recorder (Kipp & Zonen).

7.6. Measurement of substrate initial uptake rates

Cells were harvested, washed and ressuspended as stated above (Section 7.5.). To estimate the initial uptake rates of glucose, glycerol or mannitol, previously described methodologies were used [Lages and Lucas, 1995]. Aqueous solutions of [U-^{14}C] glycerol, [U-^{14}C] glucose and D-[1-^{14}C] mannitol from Amersham (England) were prepared with specific activities of 9.25 MBq $mmol^{-1}$, 5.55 MBq $mmol^{-1}$ and 9.25 MBq $mmol^{-1}$, respectively. The concentration of the cell suspension in the assays was 10 to 12 (mg dry wt) ml^{-1}. Sampling times used were 0, 5 and /or 10 sec. (the linearity of uptake was maintained for up to 20 sec.). Each experiment was repeated at least three times. Kinetic constants were estimated from Eadie-Hofstee plots and confirmed by computer nonlinear regression analysis using GraphPad PRISM (GraphPad Software, Inc.). No quenching effects were observed in uptake experiments, even in buffer containing high NaCl concentrations. Initial rates of proton uptake upon glycerol addition, in the presence or absence of NaCl, were estimated using the methodology described previously [Lages and Lucas, 1995]. All the experiments were performed at 30 °C and pH 5.0.

Parallel experiments of radiolabelled substrate uptake were performed incubating the cells for 2 min in the presence of the uncouplers carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and tetraphenylphosphonium (TPP), as well as ATPase inhibitors *N,N´*-dicyclohexylcarbodiimide (DCCD), diethylstilboestrol (DESB), 2,4-dinitrophenol (DNP) and sodium orthovanadate, according to the concentrations mentioned in the text.

The effect of other substrates over either glucose, glycerol or mannitol uptake, was assayed incubating the cells with buffer containing 200 mM of each substrate for 2 min, prior to radiolabelled solution addition. The inhibitory effect of ethanol over transport was determined incubating the cells for 2 minutes in increasing concentrations of ethanol, from 5 to 15 % (v/v), after which uptake was assayed and inhibitory constants were calculated [van Uden, 1985; 1989].

The effect of external pH on glucose, glycerol and mannitol uptake was determined incubating the cells in buffers with pH ranging from 3 to 7 (citrate adjusted). Uptake rates were also determined in the presence of 1 M NaCl.

7.7. Measurement of intracellular volume

The intracellular volume was measured in cells cultivated on glucose in the presence of different salt concentrations. This was done as previously described by Rottenberg (1979) and de la Peña and collaborators (1982), using [^{3}H] H_2O (Amersham) as total marker and [*methoxy*-^{14}C] inulin (NEN Research Products) as external marker. Measurements were made incubating salt-grown cells in buffer containing the same NaCl concentration of growth medium. Alternatively, cells cultivated in

the absence of stress were subjected to various degrees of salt shock, incubating in buffer containing different NaCl concentrations, for periods ranging from 30 min to 2.5 hours.

7.8. Measurement of radiolabelled glycerol and mannitol accumulation ratios

Radiolabelled glycerol accumulation ratios and radiolabel efflux obtained either by the action of 40 µM CCCP, 18 mM TPP, or by the addition of 100 mM glycerol or 100 mM of glucose, were determined as previously described [Lages and Lucas, 1995]. Accumulation prevention was analyzed using the same amounts of CCCP and TPP, as well as 100 mM glucose and 100 mM of glycerol-3-phosphate. The experiment was started adding 50 mM [U-^{14}C] glycerol (10 mM final concentration with 300 dpm/nmol specific activity). Radiolabelled mannitol accumulation ratios were estimated using the same methodology as for glycerol. The experiment was also started adding 50 mM D-[1-^{14}C] mannitol (278 dpm/nmol specific activity). The intracellular substrate concentrations were calculated using the value for intracellular volume estimated as described above.

7.9. Measurement of respiratory and fermentative fluxes

Cells were grown, harvested in mid exponential growth phase by centrifugation in a model 4K10 centrifuge (B.Braun, Germany), washed twice and ressuspended in ice-cold distilled water containing the same amount of sodium chloride as the growth medium. The final cell suspension had a concentration of 25 to 30 mg dry weight ml^{-1}. O_2 consumption rate was monitored using an O_2 selective electrode as described in Neves and collaborators (1997). O_2 consumption rate was expressed in µmol O_2 consumed min^{-1} g^{-1} dry wt^{-1}. CO_2 production rates were determined using a CO_2 selective electrode (Radiometer Copenhagen E5036-0). The electrode was coupled to a standard pH/mV meter (Radiometer Copenhagen PHM 82) connected to a flat recorder (Kipp & Zonen) and CO_2 liberation was monitored in a chamber with temperature control and gentle magnetic stirring. The reaction mixture contained 4.5 ml of growth medium without carbon source and 0.25 ml of cellular suspension (25 mg ml^{-1}). When the mV value stabilized, a starter of 0.25 ml of 2 M glucose was added. The concentration of CO_2 released per time was determined within the linear slope of mV variation, using a logarithmic calibration curve made according to the electrode supplier instructions. The specific CO_2 production rate was estimated indexing to biomass dry weight determinations: µmol CO_2 released min^{-1} g^{-1} dry weight.

7.10. Preparation of cell-free extracts

Cultures (100 ml) in mid exponential growth phase, with an O.D. (A_{640nm}) 0.7-0.8, were harvested as stated above and resuspended in 1 ml 10 mM triethanolamine buffer (pH 7.5) containing 1 mM dithioerytritol and 1 mM EDTA. Cells were disrupted by at least 15 cycles for 1 min hand-shaking on a vortex, followed by 1 min interval rest in ice, using 1 g of 0.5 ø mm glass beads. Supernatants were separated by centrifugation at 15 000 r.p.m. during 30 min at 4 °C, and kept on ice for a short period of time prior to the assay.

7.11. Enzyme assays

The spectrophotometric assays to determine enzyme activity were performed in a Perkin Elmer Lambda 2 UV-VIS spectrophotometer connected to an Epson FX-850 printer, in 1 ml polystyrene cuvettes. Protein concentration was determined by the method of Lowry, modified by Peterson (1977), using bovine serum albumin as standard. The production of oxidized or reduced NAD and NADP was followed measuring absorbance at 340 nm. The absorption coefficients used to calculate enzyme activity were 6.22 x 10^3 M^{-1} cm^{-1} for reduced NADH, and 6.20 x 10^3 M^{-1} cm^{-1} for reduced NADPH. In each new assay, linear increase in activity with increasing amounts of cell extract was verified. Coupling enzymes, co-factors and substrates were obtained from Sigma. In all cases it was checked whether the correspondent quantities used were not limiting the reaction. Assays were done at 25 °C.

Glycerol-3-phosphate dehydrogenase NAD^+ (EC 1.1.1.8) / Glycerol-3-phosphate dehydrogenase $NADP^+$ (EC 1.1.1.94) - Activity was determined using the method described by Adler and collaborators (1985) with some modifications. The assay mixture contained 20 mM imidazole buffer (pH 7.0), 1 mM $MgCl_2$, 200 mM NaCl and 0.2 mM NADH or NADPH. The reaction was started with 5 mM dihydroxyacetone phosphate.

Mitochondrial glycerol-3-phosphate dehydrogenase (EC 1.1.99.5) Activity was assayed using the method described by Adler and collaborators (1985). The reaction mixture contained 25 mM imidazole-hydrochloride (pH 7.5), 21 mM glycerol-3-phosphate, 0.07 mM MTT, 0.3mM phenazine methosulfate, 10 mM KCN, and 0.1% (v/v) Triton X-100. Activity was measured by phenazine methosulfate-mediated reduction of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) followed at 550 nm. An absorption coefficient for reduced MTT of 8.1 10^{-3} M^{-1} cm^{-1} was used to calculate enzyme activity.

Glycerol-3-phosphate phosphatase (EC 3.1.3.28) - Activity was determined using methods previously described [Ames, 1966; Sussman *et al*., 1981; Larsson *et al*., 1993] with some modifications. The assay mixture contained 20 mM tricine buffer (pH 7.0) and 5 mM $MgCl_2$. The reaction was started with 10 mM DL-glycerol-3-phosphate. Samples of 90 µl were withdrawn with 20 sec intervals, and immediately added to 10 µl of $HclO_4$ 50% (w/v) to stop the reaction. These samples were kept on ice until phosphate quantification. This was performed using a calibration curve made with concentrations of inorganic phosphate ranging from 0.01 to 1 mM. Each sample was treated by addition of 900 µl of a solution containing 6 parts of ammonium molibdate solution (4.2 g $(NH_4)_6.Mo_7O_{24}.4H_2O$ and 1 l H_2SO_4 0.5 M) and 1 part of 10 % (w/v) ascorbic acid solution freshly prepared. The samples were incubated at 45 °C for 20 min and then absorvance at 820 nm was read. The specific activity was determined using the slope of the curve of phosphate release per time.

Dihydroxyacetone kinase (EC 2.7.1.28*)* - Activity was measured using the method described by Babel and collaborators (1982), with some modifications. The assay mixture contained 50 mM imidazole buffer (pH 7.5), 20 mM $MgCl_2$, 0.13 mM NADH, 0.5 mM dihydroxyacetone, 10 U of glycerol-3-phosphate dehydrogenase. The reaction was started with 50 mM ATP.

Glycerol kinase (EC 2.7.1.30) – Assays were tried using the methodology described by Lin and collaborators (1962) with some modifications. The assay mixture contained 100 mM glycine buffer (pH 9.5), 20 mM $MgCl_2$, 300 mM hydrazine sulfate, 1.4 mM NAD^+, 10 mM glycerol and 10 U of glycerol-3-phosphate dehydrogenase. The reaction was started with 100 mM ATP.

Glycerol dehydrogenase/NAD^+ (EC 1.1.1.6) /Glycerol dehydrogenase/$NADP^+$ (1.1.1.72) - Activity was measured using the methodology described by Lin and Magasanik (1960) with some

modifications. The assay contained 100 mM glycine buffer (pH 9.5), 35 mM $(NH_4)_2SO_4$ and 0.2 mM NAD^+ or $NADP^+$. The reaction was started with 550 mM of glycerol.

Mannitol dehydrogenase (EC 1.1.1.67*)* - Activity was measured using the methodology described by Ueng and collaborators (1976).

Mannitol-1-phosphate dehydrogenase (EC 1.1.1.17) - Activity was measured using the methodology described by Boosaeng and collaborators (1976) with some modifications. The reaction mixture contained 50 mM Tris buffer (pH 7.0) and 0.2 mM NADH. The reaction was started with 10 mM fructose-6-phosphate.

Hexokinase (EC 2.7.1.1) - Activity was measured using the methodology described by Hirai and collaborators (1977).

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) - Activity was measured using the methodology described by Kuby and Noltmann (1966).

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) - Activity was measured using the methodology described by Maitra and Lobo (1971).

Alcohol dehydrogenase (EC 1.1.1.1) - Activity was measured using the methodology described by Postma and collaborators (1989).

Malate dehydrogenase (EC 1.1.1.37) - Activity was measured using the methodology described by Witt and collaborators (1968).

Isocitrate dehydrogenase/NAD^+ (EC 1.1.1.41) / Isocitrate dehydrogenase/$NADP^+$ (EC 1.1.1.42) - Activity was measured using the methodology described by Stueland and collaborators (1988), with some modifications. The reaction mixture contained 40 mM MOPS buffer (pH 7.5), 5 mM $MgCl_2$ and 1 mM $NADP^+$. The reaction was started with 0.5 mM isocitrate.

7.12. Verification of the *in vitro* NaCl toxicity over glyceraldehyde-3P dehydrogenase

Salt toxicity over enzyme *in vitro* assays, was performed according to Neves and collaborators (1997). The degree of activity inhibition was quantified using the equation generally used to quantify ethanol inhibition for transport systems [van Uden, 1989]. Accordingly, an exponential inhibition constant (K_i) for NaCl – K_{NaCl}, was determined, as well as the minimum concentration below which no inhibition could be detected, C_{min}.

7.13. Estimation of intracellular concentration of reduced and oxidised enzyme co-factors

Cells were cultivated as described above. The cultures were divided in two parts, submitted to, respectively, acid and alkaline extraction, after which the cell suspensions were centrifuged (13000 rpm for 10 min at 4 °C). The supernatants were used immediately for enzyme determination of NAD^+, NADH, $NADP^+$ and NADPH intracellular concentrations, using the methodologies described by Bergmeyer (1985). Results were presented using the following indexes: PNF (Phosphorilated Nucleotide Fraction) – [$NADP^+$ + NADPH] / [NAD^+ + NADH]; CRC (Catabolite Reduction Charge) – NADH / [NAD^+ + NADH]; ARC (Anabolic Reduction Charge) – NADPH / [$NADP^+$ + NADPH] [Führer *et al.*, 1979].

7.14. Estimation of Na^+ and K^+ intracellular concentration

Cultures (500 ml) were harvested by centrifugation with a model 4K10 centrifuge (B. Braun, Germany) and washed three times for 2 min at 9 000 r.p.m. with 1 M sorbitol ice-cold solution containing 4 g l^{-1} $MgCl_2$. Cells were digested with 10 ml of nitric acid, 14 ml l^{-1}, during 16 hours with gentle agitation. Cells debris was separated by centrifugation for 10 min at 4 °C. Supernatant was filtered through 0.45 μm cellulose acetate filters (Schleicher and Schuell, Germany). Samples were analyzed by Atomic Absorption Spectroscopy (AAS) in a Varian Spectra AA.250 PLUS spectrometer in absorption mode. To avoid potassium and sodium ionization, 1 g l^{-1} CsCl was added to the samples for the determination of [K^+] and 2 g l^{-1} KCl for the determination of [Na^+]. Samples duplicates were used to confirm some of the results using an Ion Coupled Plasma (ICP) Spectrometer, PU 7000 (Unicam), as in Neves and collaborators (1997). Additionally, sodium and potassium concentrations were measured by ^{39}K and ^{23}Na Nuclear Magnetic Resonance (NMR), as in Martins and collaborators (1997). We thank Prof. H. Santos and Dr. L. Martins from I.T.Q.B. Research Institute, Oeiras (Portugal) and Dr. C. Ribeiro from the Earth Sciences Department, Minho University, for, respectively, NMR and ICP ion determinations. We also want to address a special thank to Prof. T. Tavares and Dr. A. Ferraz from the Biological Engineering Department from Minho University for alllowing AAS ion determinations.