# Identification of oxygen-independent pathways for pyridine-nucleotide and Coenzyme-A synthesis in anaerobic fungi by expression of candidate genes in yeast

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**File Fig. 3\_raw.xls** : Calculated OD660nm of anaerobic growth of *S. cerevisiae* strains dependent or independent on supplementation of nicotinic acid (NA) or pantothenic acid (PA) in SMD medium containing Tween 80 and ergosterol. Worksheet “Panels A-B-C” Strains IMX585 (A), IMX2301 (*bna2*Δ *PfnadB PfnadA*) (B), and IMX2302 (*bna2*Δ *AtNADB AtNADA*) (C) transferred to medium with 2 % glucose with (+NA1) or without (-NA1) nicotinate after a carry-over phase (CO) in SMD∆nic containing 4 % glucose (displayed in a grey box in Fig. 3)). Worksheet “Panel D-E-F”Strains IMX585 (D), IMX2300-1 (*fms1* *NcadcA*) (E), and IMX2305 (*fms1* *TcPAND*)(F) transferred to medium with (+PA) or without (-PA) pantothenate after a carry-over phase (CO) in SMD∆pan containing 4 % glucose (displayed in a grey box in Fig. 3). Anaerobic condition in the chamber were maintained using a palladium catalyst and a 5 % hydrogen concentration.

### Anaerobic growth studies in shake flasks

Anaerobic shake-flask based experiments were performed in Lab Bactron 300 anaerobic workstation (Sheldon Manufacturing Inc., Cornelius, OR) containing an athmosphere of 85 % N2, 10 % CO2, and 5 % H2. Flat-bottom shake flasks of 50-mL were filled with 40 mL SMD-urea media containing 50 g L-1 glucose as carbon source, to ensure depletion of the vitamin/growth factor of interest, and 20 g L-1 glucose for the first transfer. The media was supplemented with vitamins with and without pantothenic acid or nicotinic acid as indicated and in all cases supplemented with Tween 80 and ergosterol. Sterile media was placed inside the anaerobic chamber 24 h prior to inoculation for the complete removal of oxygen traces. Traces of oxygen were continuously removed with a regularly regenerated Pd catalyst for H2-dependent oxygen removal placed inside the anaerobic chamber. Aerobic overnight shake-flask cultures on SMD-urea were used to inoculate the anaerobic shake flask without pantothenic acid or without nicotinic acid at an initial OD600 of 0.2. The cultures were cultivated at 30 °C with continuous stirring at 240 rpm on IKA ® KS 260 Basic orbital shaker platform (Dijkstra Verenigde BV, Lelystad, the Netherlands). Periodic optical density measurements at a wavelength of 600 nm using an Ultrospec® 10 cell density meter (Biochrom, Cambridge, United Kingdom) inside the anaerobic environment were used to follow the growth over time. After growth had ceased and the OD600 no longer increased the cultures were transferred to SMD-urea with 20 g L-1 glucose at an OD600 of 0.2.

**File Fig. 4\_Table4\_raw**: Data collected from anaerobic batch cultivation of IMX585 in SMD∆nic (A) and SMD∆pan (B), IMX2301 in SMD∆nic (C) and IMX2300-1 in SMD∆pan (D). All strains were pre-grown in the corresponding medium lacking one vitamin prior to inoculation in the bioreactor to avoid carry-over effects. Values for glucose , ethanol, glycerol, acetate and biomass are reported over time. Ethanol evaporation model used to calculate broth concentration is also provided. These data were used to calculate maximum specific growth rate (µmax) and yields of glycerol, biomass and ethanol on glucose in anaerobic bioreactor batch cultures of *S. cerevisiae* strains IMX585, IMX2301 and IMX2300-1 as reported in Table 4.

Each worksheet reports the data of a single batch in controlled bioreactor. Each strain was tested in duplicate and duplicates averages are reported in worksheet “Averages”.

Cultures were grown on SMD, SMD∆nic, or SMD∆pan, respectively, with 20 g L-1 glucose as carbon source (pH = 5). Growth rates and yields were calculated from the exponential growth phase. The ethanol yield was corrected for evaporation. Values represent average and mean deviation of data from independent cultures (n = 2). Carbon recovery in all fermentations was between 95 and 100%

### Anaerobic bioreactor cultivation

Anaerobic bioreactor batch cultivation was performed in 2-L laboratory bioreactors (Applikon, Schiedam, the Netherlands) with a working volume of 1.2 L. Bioreactors were tested for gas leakage by applying 0.3 bar overpressure while completely submerging them in water before autoclaving. Anaerobic conditions were maintained by continuous sparging of the bioreactor cultures with 500 mL N2 min−1 (≤0.5 ppm O2, HiQ Nitrogen 6.0, Linde Gas Benelux, Schiedam, the Netherlands). Oxygen diffusion was minimized by using Fluran tubing (14 Barrer O2, F-5500-A, Saint-Gobain, Courbevoie, France) and Viton O-rings (Eriks, Alkmaar, the Netherlands). Bioreactor cultures were grown on either SMD∆pan or SMD∆nic with ammonium sulfate as nitrogen source. pH was controlled at 5 using 2 M KOH. The autoclaved mineral salts solution was supplemented with 0.2 g L−1 sterile antifoam emulsion C (Sigma-Aldrich). Bioreactors were continuously stirred at 800 rpm and temperature was controlled at 30 °C. Evaporation of water and volatile metabolites was minimized by cooling the outlet gas of bioreactors to 4 °C in a condenser. The outlet gas was then dried with a PermaPure PD-50T-12MPP dryer (Permapure, Lakewood, NJ) prior to analysis. CO2 concentrations in the outlet gas were measured with an NGA 2000 Rosemount gas analyser (Emerson, St. Louis, MO). The gas analyser was calibrated with reference gas containing 3.03 % CO2 and N6 grade N2 (Linde Gas Benelux, Schiedam, The Netherlands).

Frozen glycerol stock cultures were used to inoculate aerobic 100 mL shake flask cultures on either SMD∆pan or SMD∆nic. After overnight cultivation at 30 °C, a second 100 mL aerobic shake-flask pre-culture on the same medium was inoculated at a starting OD660 of 1.0. During the exponential growth phase of this second pre-culture, biomass was harvested by centrifugation at 3000 g for 5 min and washed with sterile demineralized water. The resulting cell suspension was used to inoculate anaerobic bioreactors at an OD660 of 0.2.

File Table 3\_raw.xls : OD660nm data of aerobic cultures in SMD, SMD∆nic and SMD∆pan media of *S. cerevisiae* strains IMX585, IMX2292 (MATa *can1*Δ::*Spycas9*-natNT2 *URA3* *fms1*Δ), IMX2305 (MATa *ura3-52*::p*RPL12b-TcPAND*-t*TDH1* *URA3* *can1*Δ::*Spycas9*-natNT2 *fms1*∆), IMX2300-1 (MATa *ura3-52*::p*TDH3-NcadcA*-t*ENO2 URA3 can1*Δ::*Spycas9*-natNT2 *fms1*∆ Colony isolate 1), IMK877 (MATa *can1*Δ::*Spycas9*-natNT2 *URA3 bna2*∆), IMX2301 (MATa *can1*Δ::*Spycas9*-natNT2 *URA3* *bna2*∆ *sga1*::p*TDH3*-*PfnadA*-t*ENO1* p*CCW12*-*PfnadB*-t*ENO2)*, IMX2302 (MATa *can1*Δ::*Spycas9*-natNT2 *URA3 bna2*∆ *sga1*::p*TDH3*-*AtNADA*-t*ENO1* p*CCW12-AtNADB*-t*ENO2)* used to calculated displayed in Table 3*.*

### Aerobic growth studies in shake flasks

For the determination of the specific growth rate of the engineered strains under aerobic conditions, a frozen aliquot was thawed and used to inoculate a 20 mL wake-up culture that was then used to inoculate a pre-culture in a 100 mL flask. The exponentially growing pre-culture was then used to inoculate a third flask to an initial OD660 of 0.2. The flasks were then incubated, and growth was monitored using a 7200 Jenway Spectrometer (Jenway, Stone, United Kingdom). Specific growth rates were calculated from at least five time-points in the exponential growth phase of each culture. Wake-up and pre-cultures of IMX2301 and IMX2302 were grown in SMD∆nic. Wake-up and pre-cultures of IMX2300 and IMX2305 were grown in SMD∆pan while wake-up and pre-cultures of IMK877 and IMX2292 were grown in SMD.