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Arxula adeninivorans Recombinant Urate Oxidase and Its Application in the Production of Food with Low Uric Acid Content

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Key Words
Arxula adeninivorans · Food · Hyperuricemia · Urate oxidase · Uric acid

Abstract
Hyperuricemia and its symptoms are becoming increasingly common worldwide. Elevated serum uric acid levels are caused by increased uric acid synthesis from food constituents and reduced renal excretion. Treatment in most cases involves reducing alcohol intake and consumption of meat and fish or treatment with pharmaceuticals. Another approach could be to reduce uric acid level in food, either during production or consumption. This work reports the production of recombinant urate oxidase by *Arxula adeninivorans* and its application to reduce uric acid in a food product. The *A. adeninivorans* urate oxidase amino acid sequence was found to be similar to urate oxidases from other fungi (61–65% identity). In media supplemented with adenine, hypoxanthine or uric acid, induction of the *urate oxidase (AUOX)* gene and intracellular accumulation of urate oxidase (Auoxp) was observed. The enzyme characteristics were analyzed from isolates of the wild-type strain *A. adeninivorans* LS3, as well as from those of transgenic strains expressing the *AUOX* gene under control of the strong constitutive *TEF1* promoter or the inducible *AYNI1* promoter. The enzyme showed high substrate specificity for uric acid, a broad temperature and pH range, high thermostability and the ability to reduce uric acid content in food.

Introduction

Hyperuricemia is defined as serum uric acid concentration >6.8 mg/dl [Terkeltaub, 2010], a level that is above the physiological saturation of uric acid. Asymptomatic hyperuricemia can be detected in some cases and is caused by reduced renal uric acid excretion combined with increased uric acid synthesis from endogenous sources (breakdown of purines during cell turnover) or exogenous sources (food with high purine content) [Wolfram, 1992]. A minority of patients with hyperuricemia develop symptoms of gout, tophi and acute uric acid nephropathy [Wortmann, 2002], which are caused by the formation of micro- and macroscopic monosodium urate crystals. These crystals cause swelling, inflammation and pain in the joints of the lower extremities [Sorbera et al., 2010]

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and worldwide, between 1 and 2% of adults, mostly male, and often in industrialized countries, are affected by gout [Sorbera et al., 2010].

In the recent times, different treatments for gout have been developed with three main goals – treatment of acute gout attacks, prophylaxis, and long-term therapies to lower uric acid levels [Dalbeth, 2011]. In the case of acute gout attacks, patients are often treated with non-steroidal anti-inflammatory drugs and glucocorticoids for reduction of pain and treatment of inflammation [Sorbera et al., 2010] or recombinant urate oxidase-like rasburicase (Fasturtec®; Sanofi-Aventis) [Leinmüller, 2001] or pegloticase (Krystexxa®; Savient Pharmaceuticals) [Schlesinger et al., 2011] to rapidly reduce uric acid levels by converting it to allantoin which is 5–10 times more soluble. Common prophylaxis and long-term therapies are made with the xanthine oxidase inhibitors allopurinol and febuxostat or uricosuric agents such as probenecid or benzbromarone [Burns and Wortmann, 2011; Wittköpper et al., 2011].

Prevention can be achieved through lowering the uptake of exogenous uric acid by avoiding purine-rich food such as meat (especially muscle tissue, bowels and skin), certain fish (herring and shrimps) and plants (soy and other legumes), reducing alcohol and through weight loss [Wolfram, 1992]. Some of these restrictions can lead to reduction of life quality. Our novel approach for prevention of hyperuricemia is through the reduction of uric acid content of food by urate oxidase treatment, either during food production or during ingestion.

Urate oxidase (EC 1.7.3.3; urate oxygen oxidoreductase) catalyzes the oxidative opening of uric acid in the presence of oxygen. The reaction is an enzymatic oxidation combined with a non-enzymatic hydrolysis step, which produces hydrogen peroxide [Kahn et al., 1997]. The product, allantoin, is 5–10 times more soluble than uric acid and does not precipitate at physiological concentrations. Urate oxidase can also be used to analytically to determine uric acid content of serum [Liu et al., 1994; Zhao et al., 2009] or therapeutically for uric acid reduction in biological fluids for resolving hyperuricemic disorders such as tumor lysis syndrome, which can occur during chemotherapy [Wang et al., 2006].

Urate oxidase is an inducible enzyme that is found in a variety of organisms such as bacteria [Arima and Nose, 1968; Lotfy, 2008], fungi [Franke and Krieg, 1952], yeast [Liu et al., 1994], algae [Alamillo et al., 1991], plants [Montalbini et al., 1997] and is expressed in mammalian peroxisomes in specific tissues [Antonenkov and Panchenko, 1978; Rajoka et al., 2006]. It is however absent in higher primates (hominoids and some New World monkeys), birds, terrestrial reptiles and most insects [Keebaugh and Thomas, 2010; Keilin, 1959; Oda et al., 2002]. The lack of functional urate oxidase in higher primates is a consequence of independent mutations in the coding sequence of the *urate oxidase* gene [Oda et al., 2002; Wu et al., 1992] and results in uric acid as the final purine degradation product in these organisms.

In this study we present the construction of an *Arxula adeninivorans* strain that overexpresses the *urate oxidase (AUOX)* gene and produces high concentrations of urate oxidase, and its application for the reduction of uric acid in food. The *AUOX* gene was isolated from the yeast *A. adeninivorans* (syn. *Blastobotrys adeninivorans*) [Kurtzmann and Robnett, 2007], an arthroconidial, xerotolerant, anamorphic, non-pathogenic, non-conventional yeast [van der Walt et al., 1990], which has unusual biochemical features. *A. adeninivorans* is able to assimilate and utilize a broad range of substrates, including purines such as uric acid, adenine and hypoxanthine [Middelhoven et al., 1984], *n*-alkanes or starch as sole energy and carbon source [Gienow et al., 1990] and nitrate as sole nitrogen source [Böer et al., 2009c]. Additionally, the yeast is thermo- and halotolerant [Wartmann et al., 1995; Yang et al., 2000]. It can be cultured up to 48°C and up to 20% NaCl. Above 42°C the morphology changes from the yeast-budding cells to mycelia which are dependent on an altered gene expression pattern [Wartmann et al., 1995]. All of these properties support the potential of *A. adeninivorans* as an ideal host for homo- or heterologous gene expression and as source for genes that have biotechnological significance.

Results

Isolation and Characterization of the AUOX Gene

The putative nucleotide sequence of the *AUOX* gene was determined from the complete *A. adeninivorans* sequence and annotated genome [in preparation]. It contains an ORF of 921 bp with a conserved potential TATA element in the 5′-untranslated region (78 bp upstream of the proposed initiation codon). The ORF encodes a protein of 306 amino acids with a molecular mass of 34.7 kDa and an isoelectric point of 7.02. Auoxp shows significant identity with other fungal urate oxidases, including *Neosartorya fischeri* (65%), *Neurospora crassa* (62%), *Ajellomyces dermatitidis* (61%), *Penicillium marneffei* (61%), *Talaromyces stipitatus* (61%), *Gibberella fujikuroi* (61%) and some *Aspergillus* species (61–64%).

Nitrogen Source-Dependent Expression of the AOUX Gene

The regulation of *AUOX* transcription was studied by qRT-PCR with *Arxula* genes *AHBS4*, *ALG9*, *TEF1* and *TFIID* as stably expressed endogenous references. *A. adeninivorans* LS3 cells cultured in yeast minimal medium (YMM)-glucose supplemented with 19 mM $NH_4H_2PO_4$ as nitrogen source for 24 h at 30°C were shifted to YMM-glucose containing 5 mM adenine, 5 mM hypoxanthine or 5 mM $NaNO_3$ as nitrogen sources and monitored for *AUOX* transcript accumulation for 24 h at 30°C. Immediately after the shift from YMM-glucose-$NH_4H_2PO_4$ to YMM-glucose-adenine and YMM-glucose-hypoxanthine, the *AUOX* transcript level, calculated as transcript concentration in YMM-glucose-purine minus transcript concentration in the control YMM-glucose-$NaNO_3$, increased and reached a maximum at 4 h. The maximal transcript accumulation was 2-fold higher in yeast cells cultured in YMM-glucose supplemented with 5 mM hypoxanthine than in YMM-glucose-adenine. After 4 h the transcript level decreased to the basal level (fig. 1a).

Urate oxidase (Auoxp) activity, produced in the same cultivation conditions, was determined. These investigations demonstrated a strong increase of enzymatic activity immediately after the shift from YMM-glucose-$NH_4H_2PO_4$ to YMM-glucose-purine whereas the negative control (YMM-glucose-$NH_4H_2PO_4$) showed no increase in Auoxp. The maximal Auoxp activity was achieved 8 h after the shift to the uric acid medium and after 12 h for the adenine and hypoxanthine media, after which it decreased to the basal level over the next 36 h (fig. 1b).

In parallel, *AUOX* transcript accumulation relative to purine concentration in YMM-glucose was assessed. *A. adeninivorans* LS3 cells were harvested 2 h after a shift to YMM-glucose supplemented with 0.5, 1, 3, 4 and 5 mM uric acid, hypoxanthine or adenine or $NaNO_3$ as a control. The highest *AUOX* transcript accumulation was achieved in presence of 5 mM uric acid. Lower inducer concentrations (0.5 and 1 mM) resulted in lower *AUOX* transcript levels. Furthermore, inducer concentrations >3 mM caused an increased accumulation of mRNA in the cells (fig. 1c).

Urate oxidase activity was also analyzed. The enzyme activity was too low for analysis at 2 h after the shift to YMM-glucose with varying uric acid, hypoxanthine and adenine concentrations and consequently the yeast cells were harvested at 4 h. Under these conditions the urate oxidase activity increased up to 1 mM purine and stayed relatively constant to 5 mM for each inducer. In contrast, cells cultured in YMM-glucose-$NH_4H_2PO_4$ showed only weak urate oxidase activity which was independent of the $NH_4H_2PO_4$ concentration (fig. 1d).

Production of Recombinant Urate Oxidase, Auoxp

The Xplor®2 platform has been established as a routine transformation/expression platform in *A. adeninivorans* [Böer et al., 2009b]. The system, which allows construction of resistance marker-free yeast transformants, is based on a bacterial vector backbone into which yeast modules for selection and expression can be inserted between two 25S rDNA fragments that are arranged in the same orientation but in reverse order relative to the genomic rDNA. To use the Xplor®2 platform for construction of transgenic Auoxp strains, the ATRP1m selection marker module with one or two expression modules for Auoxp and either with or without N- or C-terminal fused HisTag, were inserted into the Xplor2 vector between the 25S rDNA fragments. Yeast rDNA integrative expression cassettes (YRCs) were produced by digestion with *Asc*I and yeast integrative expression cassettes (YICs) were produced by digestion with *Sbf*I.

Both types of linear cassette (fig. 2) were successfully transformed into *A. adeninivorans* G1212 and between 100 and 200 transformants were obtained per microgram of transforming cassette. The resulting transgenic YRC/YIC *A. adeninivorans* strains carried one or two AUOX expression modules with constitutive *TEF1* or inducible *AYNI1* promoters in front of the *AUOX* gene and with or without the 5′- or 3′-fused HisTag coding region (data not shown). After stabilization of the transgenic yeast strains by passaging on a non-selective medium for 30 days (as described in the Materials and Methods section), there was no loss of transgenic strains during the cultivation.

Cells for initial analysis of recombinant Auoxp accumulation were grown in YMM-glucose-$NaNO_3$ at 30°C for 24 h. It was expected that because Auoxp does not contain an obvious secretion sequence, the recombinant protein would be localized intracellularly. Protein extracts (from approx. 50 transformants per construct) were consequently analyzed for the presence of recombinant Auoxp activity (data not shown).

Transgenic yeast strains with the highest recombinant Auoxp activities per integration cassette were then analyzed in time-course experiments. The strains listed in table 1 were cultured in YMM-glucose-$NaNO_3$ for 96 h at 30°C. This medium allows high-level expression from the *TEF1* and *AYNI1* promoters with cultures entering the stationary growth phase after approximately 24 h. Cell

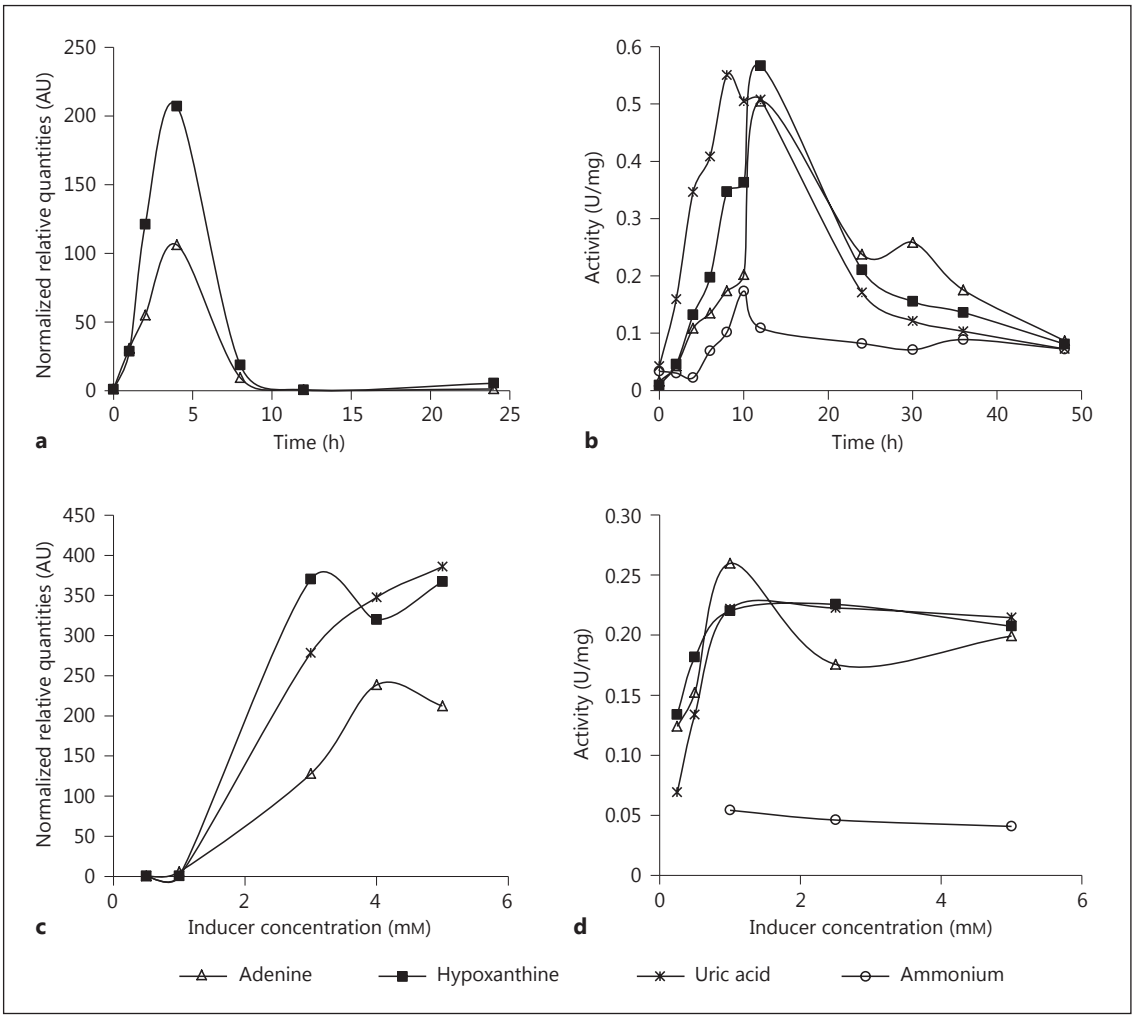


Fig. 1. Influence of the nitrogen source on *AUOX* transcript accumulation (**a**, **c**) and urate oxidase activity (**b**, **d**). *A. adeninivorans* LS3 was cultured for 24 h at 30°C in YMM-glucose supplemented with 19 mM $NH_4H_2PO_4$ and then shifted to YMM-glucose supplemented with different nitrogen sources in different concentrations and cultured for a further 24 h. The *AUOX* transcript was analyzed by qRT-PCR with the *Arxula* genes, *AHBS4*, *ALG9*, *TEF1* and *TFIID* used as stable expressed endogenous references (**a**) and 48 h for the detection of the urate oxidase activities (**b**). The yeast cells were shifted in YMM-glucose supplemented with (**a**) 5 mM adenine, hypoxanthine and $NaNO_3$, (**b**) 5 mM adenine, hypoxanthine, uric acid and $NH_4H_2PO_4$, (**c**) 0.5, 1, 3, 4 and 5 mM adenine, hypoxanthine or $NaNO_3$ for 2 h, (**d**) 0.25, 0.5, 1, 2.5 and 5 mM adenine, hypoxanthine, uric acid or $NH_4H_2PO_4$ for 4 h. Normalized relative quantities are calculated using the model of Hellemans et al. [2007] by adjusting for differences in PCR efficiency between the gene of interest and multiple reference genes.

extracts and aliquots of the culture medium were then analyzed for urate oxidase activity.

In contrast to the negative control strains (activity approx. 5 U/l culture), all transformants with AUOX expression modules exhibited an intracellularly localized urate oxidase. Most cells achieved maximum Auoxp levels within approximately 24 h (between 55 and 81 U/l culture), before declining to a basal level at 96 h. Maximum activity (70–90 U/l culture) was achieved in *A. adeninivorans* strains G1212/YIC102-AYNI1-AUOX, G1212/YRC102-AYNI1-AUOX, G1212/YIC102-TEF1-AUOX-6H and G1212/YRC102-TEF1-AUOX-6H (24 h). During 96 h of cultivation, the urate oxidase activity in YRC/YIC transformants with integrated AYNI1-AUOX expression module decreased slightly. Only the YRC/YIC transformants with TEF1-AUOX-6H expression module exhibited increased urate oxidase levels at the end of the 96-hour cultivation. A correlation between

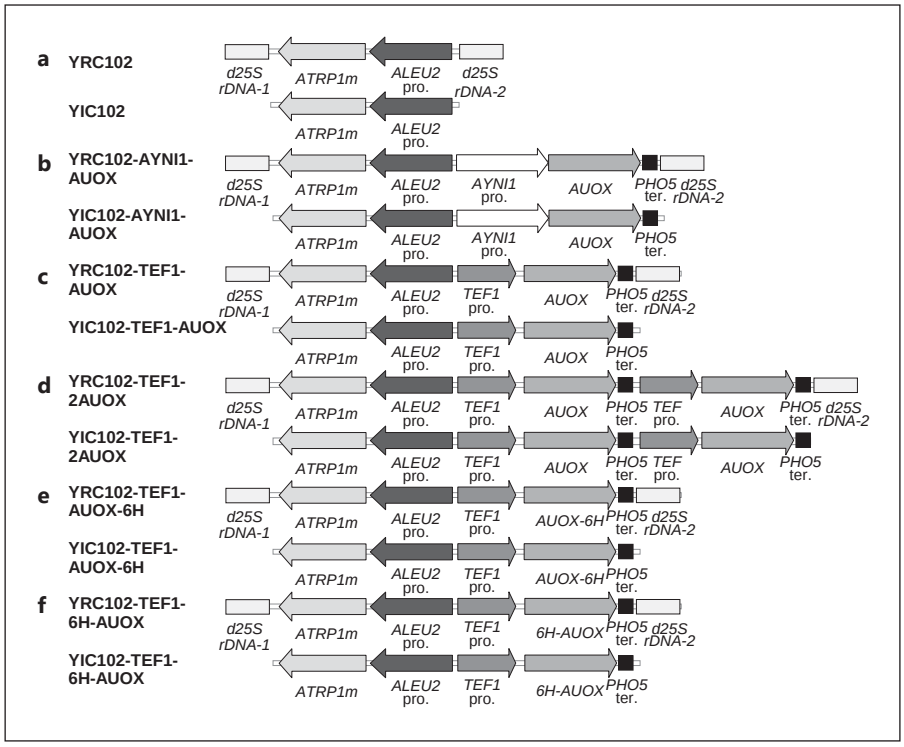


Fig. 2. Physical maps of yeast integration-expression cassettes (YRC and YIC) used in this study. The selection module contains the selection marker *ATRP1m* fused to the *ALEU2* promoter. The selection module is fused to none (**a**), one (**b**, **c**, **e**, **f**) or two expression modules (**d**). The expression modules contain the following elements: *A. adeninivorans*-derived constitutive *TEF1* (**c–f**) or inducible *AYNI1* (**b**) promoter, *AUOX*-coding sequence *(AUOX)* (**b–f**) and *S. cerevisiae*-derived *PHO5* terminator (**b–f**). *AUOX*-coding sequences are fused to C-terminal HisTag (**e**) or N-terminal HisTag (**f**) encoding sequences. YRCs (*Asc*I fragments) are flanked by 25S rDNA sequences for targeting. YICs without 25S rDNA targeting sequences were obtained as *Sbf*I fragments.

Table 1. Maximum yields of urate oxidase activity obtained in recombinant strains of *A. adeninivorans*

Yeast strain	Auoxp activity, U/l culture		Dry cell weight, g dcw/l culture		Yield coefficient (P/X), U/g dcw	
	24 h	96 h	24 h	96 h	24 h	96 h
G1212/YIC102	5.3	5.5	3.8	4.4	1.4	1.2
G1212/YIC102-AYNI1-AUOX	81.3	68.3	3.3	4.7	25.2	14.5
G1212/YIC102-TEF1-AUOX	76.8	44.6	3.8	4.6	20.6	9.6
G1212/YIC102-TEF1-2AUOX	58.7	35.0	3.9	4.9	15.0	7.2
G1212/YIC102-TEF1-AUOX-6H	70.3	86.2	3.9	5.1	18.4	16.9
G1212/YIC102-TEF1-6H-AUOX	66.9	51.0	3.4	4.8	19.5	10.8
G1212/YRC102	5.4	4.8	3.7	4.7	1.5	1.0
G1212/YRC102-AYNI1-AUOX	81.1	81.5	3.2	4.6	26.0	17.6
G1212/YRC102-TEF1-AUOX	54.9	31.0	3.7	4.8	14.8	6.6
G1212/YRC102-TEF1-2AUOX	76.2	31.5	3.9	4.5	19.7	7.1
G1212/YRC102-TEF1-AUOX-6H	80.3	84.5	4.0	4.9	20.0	17.2
G1212/YRC102-TEF1-6H-AUOX	61.0	35.3	4.0	4.9	15.5	7.2

the activity levels of YRC and YIC transformants or the number of AUOX expression modules was not detectable; however, the maximum yield coefficients (Y[P/X]) measured in G1212 strains with TEF1-AUOX-6H expression modules were up to 30% higher than in the transformants containing the TEF1-AUOX or TEF1-6H-AUOX expression modules (table 1; fig. 3).

Parameters of Urate Oxidase

The endogenous urate oxidase was purified to homogeneity by fractionated ammonium sulfate precipitation, followed by DEAE-Sepharose ion exchange chromatography and gel filtration on a Superdex™ 200 column, as described in the Materials and Methods section. The final yield and specific activity were 1% and 0.65 U/mg protein,

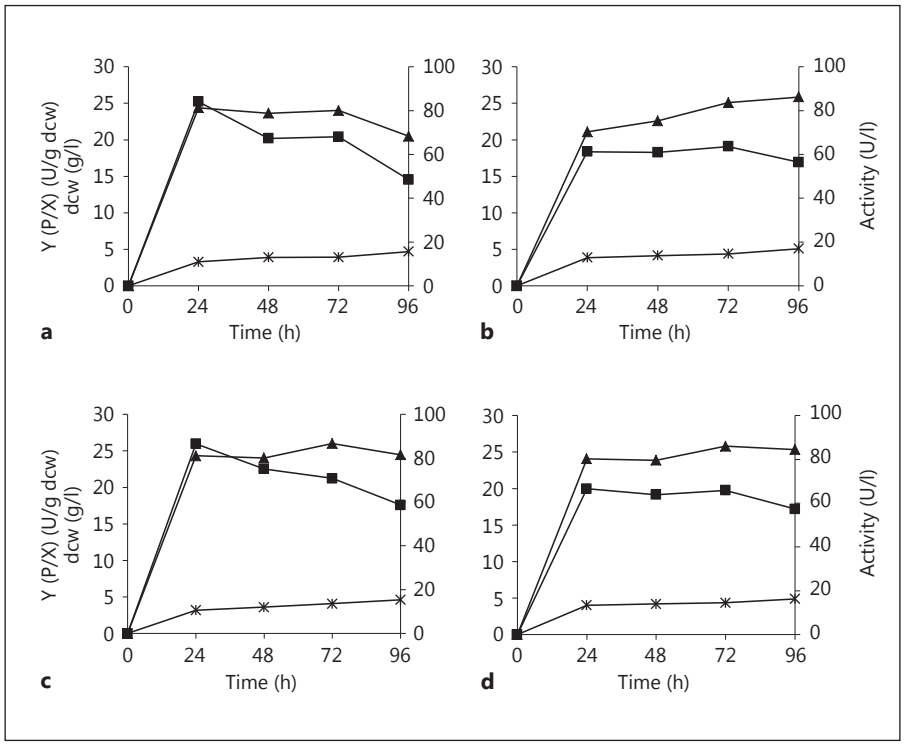
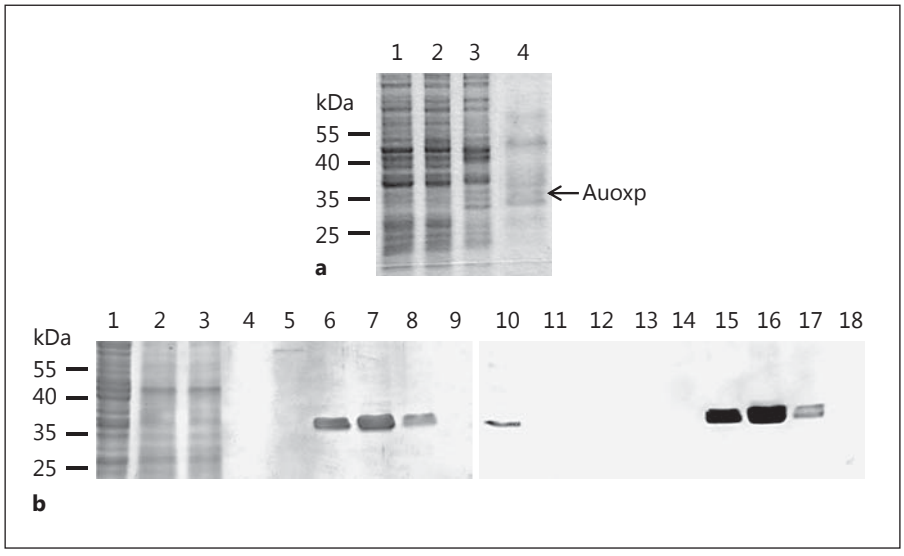


Fig. 3. Time courses of expression of urate oxidase by transgenic strains of *A. adeninivorans*. Transformants G1212/YIC102-AYNI1-AUOX (**a**), G1212/YIC102-TEF1-AUOX-6H (**b**), G1212/YRC102-AYNI1-AUOX (**c**) and G1212/YRC102-TEF1-AUOX-6H (**d**) were cultured in shake flasks for 96 h at 30°C in the presence of YMM-glucose-$NaNO_3$. At the indicated time, 2-ml aliquots of the culture were assayed for intracellular urate oxidase activity (activity in U/l, triangle) and to determine biomass (dcw in g/l, asterisk) as described in the Materials and Methods section, and to calculate the urate oxidase output (Y(P/X) in U/g dcw, square).

Fig. 4. SDS-PAGE analysis of purified endogenous Auoxp (**a**) and recombinant Auox6hp (**b**) from *A. adeninivorans* LS3 and G1212/YRC102-TEF1-AUOX-6H. **a** *A. adeninivorans* LS3 cells were cultured for 24 h at 30°C in YMM-glucose-$NaNO_3$ and then shifted to 5 mM YMM-glucose-adenine for 6 h. After harvesting and disrupting the yeast cells, the crude extract (1) was purified by fractionated ammonium sulfate precipitation (2), DEAE ion exchange chromatography (3), Superdex™ 200 chromatography (4) and then electrophoresed on 12% SDS-PAGE. **b** *A. adeninivorans* G1212/YRC102-TEF1-AUOX-6H cells were cultured for 24 h at 30°C in YMM-glucose-$NaNO_3$, harvested, crude extracts prepared (1, 10) and purified by Ni-NTA chromatography. The following purification steps flow-through 1st (2, 11), flow-through 2nd (3, 12), washing with 5 mM imidazole (4, 13), washing with 100 mM imidazole (5, 14), eluate 1 (6, 15), eluate 2 (7, 16), eluate 3 (8, 17), eluate 4 (9, 18) (all eluation steps with 1 ml 1 M imidazole) were electrophoresed on 12% SDS-PA gels (1–9) and analyzed by immunoblot analysis with anti HisTag antibodies (10–18).



respectively. A protein band with an apparent molecular mass of 35 kDa was detected by SDS-PAGE (fig. 4a). This band was cut out and the protein was digested with the endopeptidase trypsin. The resulting peptides were analyzed by MALDI-TOF and ESI-Q-TOF mass spectrometry, which revealed an amino acid overlay of 37% with the putative amino acid sequence of the *A. adeninivorans* urate oxidase Auoxp (data not shown).

In parallel, the recombinant Auox6hp was isolated and purified from *A. adeninivorans* G1212/YRC102-TEF1-AUOX-6H by Ni-NTA His Bind Resin column chromatography (fig. 4b) and by the method used for the endogenous urate oxidase. The endogenous Auoxp and recombinant urate oxidases Auox6hp exhibited similar characteristics. Both enzymes were shown to have a molecular mass of approximately 67 kDa by analytical gel

Table 2. Properties of the endogenous and recombinant urate oxidases synthesized in *A. adeninivorans* LS3 and G1212/YRC102-TEF1-AUOX-6H

Property	Endogenous urate oxidase	Recombinant urate oxidase
pH optimum*	8.0	9.5
pH stability*	7.5–9.5	8.0–10.0
Temperature optimum*, °C	40	35
Temperature stability, °C	up to 50	up to 50
Michaelis-Menten constant (K_m), μM	56	71
k_{cat}, 1/s	38	121
k_{cat}/K_m, 1/μM s	0.68	1.71
M_r native, Da	70,000	70,000
M_r denatured, Da	34,700	35,500

The Michaelis constant was estimated using Hanes-Woolf plot. pH stability was defined as the range and thermal stability as highest temperature at which over 80% of the initial activity was retained after incubation time of at least 60 min.

* Addition of 2 mM DTT (final concentration).

Table 3. Effects of various cations and inhibitors on the stability and activity of the endogenous and recombinant urate oxidase

Compound	Concentration, mM	Endogenous urate oxidase, %	Recombinant urate oxidase, %
Control (without compound)	–	100	100
$CaCl_2$*	1/0.1	67/101	56/112
$CoCl_2$*	1/0.1	18/123	4/134
$CuCl_2$*	1/0.1	9/8	0/4
$CuSO_4$*	1/0.1	5/8	0/3
$FeCl_3$*	1/0.1	85/85	109/112
$MgCl_2$*	1/0.1	90/95	119/129
NaCl*	1/0.1	94/99	127/108
$NiCl_2$*	1/0.1	29/97	24/121
$NiSO_4$*	1/0.1	28/100	26/109
NH_4Cl*	1/0.1	95/98	123/104
ZnCl*	1/0.1	25/108	47/114
$ZnSO_4$*	1/0.1	20/103	24/107
EDTA	1/0.1	112/110	110/106
$HgCl_2$	1/0.1	0/11	4/7
1,10-Phenanthroline	1/0.1	86/103	87/99
DTT	1/0.1	151/150	127/126
L-Cysteine	1/0.1	144/151	119/129
PMSF	1/0.1	103/104	112/107

The enzyme was incubated for 1 h at 37° C in potassium phosphate buffer pH 7.7 containing the indicated concentrations of listed supplements. The residual activity was measured with standard enzyme assay (UV measurement) as described in the Material and Methods section. * Contained 2 mM L-cysteine.

filtration, while forming a distinct band at approximately 35 kDa on denaturing SDS-PA gels. These data indicate that the active enzyme is probably a homodimer.

Enzymatic Analysis of Endogenous and Recombinant Urate Oxidase

Both urate oxidases exhibit similar biochemical parameters with pH and temperature optima of 8.0 and 40°C for the endogenous Auoxp and 9.5 and 35°C for the recombinant Auox6hp with C-terminal HisTag. Both proteins had similar urate oxidase activity over a broad temperature and pH range (Auoxp 25–40°C, pH 7.5–9.5; Auox6hp 30–45°C, pH 8.0–10.0). The residual enzyme activity after incubation for 2 h at 37°C was constant at pH 7.5–9.5 (Auoxp) and at pH 8.0–10.0 (Auox6hp). In addition, both urate oxidases have high thermostabilities with incubations for 1 h at 50°C reducing the residual activity of both enzymes by less than 10%. The K_m value of the purified urate oxidase was determined by Hanes-Woolf plot and was approximately 56 μM for the endogenous and 71 μM for the recombinant urate oxidase (table 2).

Various metal ions and reagents were assayed for their effects on urate oxidase activity. The enzyme was completely inactivated in the presence of 1.0 mM Cu^{2+} and Hg^{2+}. The residual activity in the presence of 1 mM Zn^{2+}, Co^{2+} and Ni^{2+} was less than 25% and in the presence of 1 mM Ca^{2+} the residual activity was 50%, whereas Fe^{3+}, Mg^{2+}, Na^+, NH_4^+, EDTA, 1,10-phenanthroline and phenyl methyl sulfonyl fluoride (PMSF) had weak or no effect on the endogenous urate oxidase and only weak activating effects on the recombinant Auox6hp, whereas dithiothreitol and L-cysteine increased the enzymatic activity (table 3).

The substrate specificity of the enzyme was determined using a variety of uric acid analogues and purine derivates. Only uric acid was converted to H_2O_2 and allantoin which was determined using (A) the H_2O_2 quantification method and (B) absorbance spectra differences between of 230 and 330 nm during incubation for 1 h at 37°C (data not shown). The addition of uric acid analogues, 8-azaxanthine and oxonic acid, caused a decrease of enzymatic activity to less than 25%. All other analogues or purine derivates that were tested exhibited only weak effects or had no effect (table 4).

Use of Urate Oxidase to Reduce Uric Acid in Beef Stock

Investigation of the application of urate oxidase Auox6hp for the reduction of uric acid in food was done

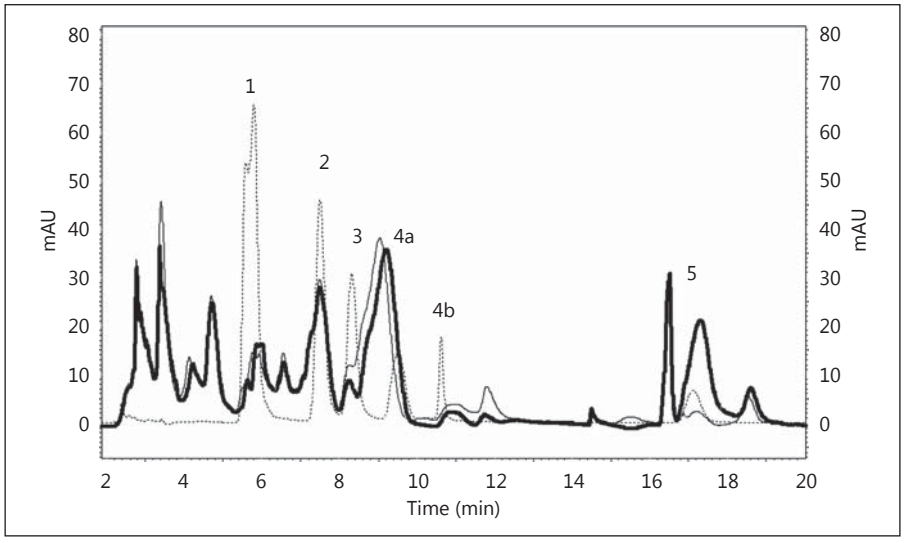


Fig. 5. HPLC analysis: 0.5 mg/ml stock solutions of the purine standards were used for determination of their retention times (dashed line) with adenine (1), hypoxanthine (2), xanthine (3), guanine (4a, b) and uric acid (5). The profile of the beef stock solution with additional uric acid and 0.2 U/ml recombinant urate oxidase Auox6hp without incubation time (thick line) and after 60 min at 40°C (thin line).

Table 4. Effects of purine derivates on the endogenous and recombinant urate oxidase activity

Purine	Endogenous urate oxidase activity, %	Recombinant urate oxidase activity, %
Control (without additional purine)	100	100
Adenine	111	108
Adenosine	105	103
Allantoin	102	99
8-Azaxanthine	22	26
Caffeine	102	92
Guanosine	111	100
Hypoxanthine	116	104
Inosine	106	100
Oxonic acid potassium salt	9	18
Theobromine	113	100
Xanthine	82	66
Xanthosine	103	98

The enzyme reaction was performed under standard conditions (H_2O_2 measurement), except that the purine derivates were added additionally in the same concentration as uric acid to the reaction buffer. The residual activity was measured as described in the Materials and Methods section.

with beef stock (50 mg/ml REWE Bio Rinderbrühe; REWE, Germany). It contains 0.2 mg/l uric acid, 1.8 mg/l xanthine, 3.4 mg/l adenine, 13.4 mg/l hypoxanthine and 35.2 mg/l guanine. Only allantoin was not detectable with the HPLC system. However, the uric acid content of this product is relatively low and it was supplemented with additional uric acid to provide a realistic investigation. After incubation for 60 min at 40°C with 0.2 U/ml urate oxidase, the beef stock was analyzed for uric acid by HPLC. The control, beef stock with 0.2 U/ml urate oxidase, without incubation, contained 28.7 mg/l uric acid. In contrast, the concentration in the sample incubated with urate oxidase at 40°C for 60 min was 0.3 mg/l (fig. 5).

Discussion

Hyperuricemia, and its symptoms, gout, tophi and acute uric acid nephropathy, is widespread in industrial countries (1–2% of adults) [Sorbera et al., 2010]. Reduced renal uric acid excretion and increased uric acid synthesis result in increased serum uric acid concentrations. Nonsteroidal anti-inflammatory drugs are commonly used to treat the symptoms and xanthine oxidase inhibitors and uricosuric agents are given as prophylactic therapies [Burns and Wortmann, 2011; Wittköpper et al., 2011]. Additionally, avoiding purine-rich food, reduction of alcohol intake and a reduction of weight is essential for successful control of the condition [Wolfram, 1992]. We offer a new approach which involves the reduction of urate in food by treatment with urate oxidase from *A. adeninivorans* LS3 either before or during the meal and the digestion process.

The genome data from *A. adeninivorans* LS3 was used to identify the *AUOX* gene, a 921-bp ORF. The *AUOX* gene is a single copy gene as described for most organisms

[Oda et al., 2002]. Transgenic *A. adeninivorans* producer strains were constructed that accumulated high levels of recombinant urate oxidase. The Xplor®2 transformation/expression platform [Böer et al., 2009b], previously used to transform *A. adeninivorans* and other yeasts to synthesize biotechnologically interesting enzymes such as Alip1p [Böer et al., 2005], Atan1p [Böer et al., 2009a] and RR-ADH [Giersberg et al., 2012], was used to express the *AUOX* gene in *A. adeninivorans*. The *AUOX* gene was integrated into the genome of the yeast as described in Böer et al. [2009b, 2011].

A. adeninivorans YIC and YRC strains with one or two expression modules containing the *AUOX* gene, with and without 3′- or 5′-fused HisTag encoding region, were compared for accumulation of recombinant Auoxp. YRC/YIC strains with inducible *AYNI1* promoter achieved the highest productivity (G1212/YIC102-AYNI1-AUOX – 25.2 U/g dcw; G1212/YRC102-AYNI1-AUOX – 26.0 U/g dcw) at the end of the exponential growth phase (24 h). However, using the constitutive *TEF1* promoter and the *AUOX* gene flanked by HisTag encoding sequences at the 3′-end achieved 18.4 U/g dcw (G1212/YIC102-TEF1-AUOX-6H) and 20.0 U/g dcw (G1212/YRC102-TEF1-AUOX-6H) at end of the exponential growth phase (24 h). The results show an increase in Auoxp stability with C-terminal HisTag fusions compared with an N-terminal HisTag and Auoxp without HisTag. In contrast, only small differences were detected in the maximal yield coefficients between transgenic yeast strains with one or two AUOX expression modules integrated into the genome. This is similar to the transgenic *A. adeninivorans* RR-ADH producers in which an increased number of expression modules resulted in most transgenic strains with two or more expression modules showing no increase in yield, although two transgenic strains did produce higher yield coefficients [Giersberg et al., 2012]. In contrast, recombinant tannase producers with two expression modules exhibited reduced maximal yield coefficient [Böer et al., 2011]. Cultivation in fed-batch fermentation mode may enhance the production of Auoxp by transgenic *A. adeninivorans* strains as was demonstrated for tannase [Böer et al., 2011].

The regulation of the purine degradation pathway has been described for *Aspergillus nidulans* [Scazzocchio and Darlington, 1968] and *Candida* sp. [Liu et al., 1994] using enzyme activity assays. They found that directly after the addition of purines, such as adenine and uric acid, the respective genes are activated. This situation was confirmed in the purine degradation experiments in *A. adeninivorans* LS3. The *AUOX* transcript accumulation reached a maximum level at 4 h, whereas an increase Auoxp activity level was delayed 4–8 h. The reasons for this effect could be higher stability of Auoxp compared to the *AUOX* transcripts, which prevents intracellular uric acid accumulation during the degradation of adenine and other purines. The induction of *AUOX* transcript accumulation or Auoxp activity was dependent of the inducer concentration. This effect was not described in further works.

The 67-kDa Auoxp comprises two subunits with molecular sizes of about 35 kDa. Known urate oxidase subunits range from 30 to 80 kDa. Different enzyme structures have been described: monomer [Liu et al., 1994], dimer [Lucas et al., 1983], homotetramer [Giffard et al., 2011; Nishimura et al., 1982] and heterotetramer [Bongaerts et al., 1978].

Partially purified endogenous Auoxp showed optimum activity at pH 8.0 and 40°C which are properties shared with urate oxidases from *Aspergillus flavus* [Li et al., 2006], *Bacillus subtilis* [Pfrimer et al., 2010] and *Candida utilis* [Liu et al., 2011]. However, Auox6hp showed an increase in pH optimum to pH 9.5. The K_m values indicate a high affinity of urate oxidase for uric acid as substrate and are comparable with *C. utilis* (33.7 μM) and *Bacillus fastidiosus* (180 μM) [Bongaerts et al., 1978; Liu et al., 2011]. The reason for differences in pH, temperature profiles and kinetic parameters of Auox6hp may be caused by the C-terminal HisTag which can have influence on the protein structure and protein charge [Votchitseva et al., 2006]. The enzymatic activity of both recombinant and endogenous urate oxidase was stable up to 50°C for at least 1 h. This physicochemical property will be useful in using urate oxidase to treat heated food products.

Auoxp was inactivated in presence of Cu^{2+} or Hg^{2+} which was also observed in the urate oxidases of *Microbacterium* sp., *C. utilis* and *Arthrobacter globiformis* [Kai et al., 2008; Nishimura et al., 1982; Suzuki et al., 2004]. This effect was not however detected in *B. fastidiosus* [Bongaerts et al., 1978]. The increase in enzymatic activity seen with dithiothreitol and L-cysteine has also been reported for *C. utilis* urate oxidase [Nishimura et al., 1982]. These results suggest that sulfhydryl groups could be located close to the active site of the enzyme or be of significance in conformation of the protein structure.

Recombinant Auoxp, synthesized in C-terminal fusion with HisTag as Auox6hp in transgenic *A. adeninivorans* strains, was used to reduce uric acid concentration in a food product and demonstrates the potential of the enzyme as part of a new strategy to prevent hyperurice-

Table 5. PCR and qRT-PCR primers used in the construction of the plasmids pBS-AYNI1-AUOX-PHO5-SS, pBS-TEF1-AUOX-PHO5-SS, pBS-TEF1-6H-AUOX-PHO5-SS, pBS-TEF1-AUOX-6H-PHO5-SS and Xplor2.2-TEF1-2AUOX and in the calculation of *AUOX* transcript accumulation in *A. adeninivorans* LS3

Name	Nucleotide sequence	Position in gene sequence or plasmid
AUOX5	5′-ATGTAGAATTC**ATGTCCCATCTCTCTGCTGC**-3′	1–20
AUOX3	5′-AATGCGGCCGC**TTAGAGCTTAGCAGTGTTACGGGTGACAG**-3′	921–892
AUOX5-His	5′-ATTTGGAATTC**ATG***CATCATCACCATCACCAT***TCCCATCTCTCTGCTGCCCGTTACGG**-3′	1–47
AUOX3-His	5′-TTCACGCGGCCGC**TTA***ATGGTGATGGTGATGATG***GAGCTTAGCAGT**-3′	939–892
TEF1-*Spe*I	5′-GTCGAGACTAGT**CTCGACTTCAATCTATAATC**-3′	899–918 [pBS-TEF1-AUOX-PHO5-SS]
PHO5-*Spe*I	5′-TTATTACTAGT**CGGCCCCAGCTTGCATGCCTG**-3′	2306–2286 [pBS-TEF1-AUOX-PHO5-SS]
AUOX-fw-rtPCR	5′-**GAACACTGGCAAGAACGC**-3′	828–845
AUOX-rv-rtPCR	5′-**CTTAGAGCTTAGCAGTGTTACG**-3′	922–901
AHSB4-fw-rtPCR	5′-**CTCATTTATGAGGAGACCCG**-3′	200–219
AHSB4-rv-rtPCR	5′-**ATAAAGAGTTCGTCCCTGTC**-3′	349–330
ALG9-fw-rtPCR	5′-**CATGGGCCAAGGTATACTG**-3′	1502–1520
ALG9-rv-rtPCR	5′-**AGAATGCGACCGATACCA**-3′	1568–1551
TEF1-fw-rtPCR	5′-**CTCTTGGACGATTCGCC**-3′	1253–1269
TEF1-rv-rtPCR	5′-**CCGTTACCGACAATCTATTT**-3′	1398–1379
TFIID-fw-rtPCR	5′-**AGTTTGTGTCTGATATTGCCTC**-3′	500–521
TFIID-rv-rtPCR	5′-**GAAGAGCTTGCTACCGAACT**-3′	575–556

*Eco*RI (GAATTC), *Not*I (GCGGCCGC) and *Spe*I (ACTAGT) restriction sites are underlined, the sequences corresponding to the ORF are given in bold type. The nucleotide position is given relative to the first nucleotide of the respective ORF.

mia. The strategy will require enzymes that degrade purines and purine derivatives such as hypoxanthine to be commercially available to ensure complete removal of the molecules that cause hyperuricemia.

Materials and Methods

Strains, Media and Cultivation

Escherichia coli XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F′ proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})]) obtained from Stratagene (La Jolla, Calif., USA), served as the host strain for bacterial transformation and plasmid isolation. The strain was grown on LB medium supplemented with ampicillin (100 μg/ml) or kanamycin (50 μg/ml) for selection.

The yeast wild-type strain *A. adeninivorans* LS3, isolated from wood hydrolysates in Siberia (Russia) [Gienow et al., 1990] and deposited as *A. adeninivorans* SBUG 724 in the strain collection of the Department of Biology of the University of Greifswald, and the auxotrophic mutant *A. adeninivorans* G1212 [*aleu2 atrp1::ALEU2*] [Steinborn et al., 2007], were used in this study. All yeast strains were grown up to 96 h at 30°C under non-selective conditions in a yeast-extract-peptone-medium (YEPD) or under selective conditions in a YMM supplemented with 1% (w/v) glucose (YMM-glucose) containing different nitrogen sources at various concentrations [Tanaka et al., 1967].

Agar plates were prepared by addition 1.6% (w/v) agar to the liquid media.

Isolation of the AUOX Gene and Construction of Auoxp Expression Plasmids

The potential *Arxula urate oxidase (AUOX)* gene was identified from the complete sequenced and annotated genome data of *A. adeninivorans* LS3 [in preparation] and the *AUOX* nucleotide sequence has been submitted to the GenBank/EMBL data libraries (HF558665).

To express the *AUOX* gene in *A. adeninivorans* G1212, the ORF was amplified by PCR from chromosomal DNA of *A. adeninivorans* LS3, using primers that incorporated flanking *Eco*RI and *Not*I restriction sites (AUOX5/AUOX3; table 5). The amplified *Eco*RI-*AUOX-Not*I gene fragment, which corresponds to the complete ORF, was inserted into the plasmids pBS-TEF1-PHO5-SS and pBS-AYNI1-PHO5-SS (both flanked by *Spe*I and *Sac*II) between the *A. adeninivorans*-derived constitutive *TEF1* or inducible *AYNI1* promoter and the *S. cerevisiae*-derived *PHO5* terminator [Arima et al., 1983; Böer et al., 2009c].

Plasmids were constructed with single AUOX expression modules, the *TEF1* promoter – *AUOX* gene – *PHO5* terminator and *AYNI1* promoter – *AUOX* gene – *PHO5* terminator fragments. These fragments were flanked by *Spe*I and *Sac*II restriction sites and were inserted into the plasmid Xplor2.2 [Böer et al., 2009b] to generate the *Arxula* plasmids Xplor2.2-TEF1-AUOX and Xplor2.2-AYNI1-AUOX. Here the selection marker module (*ALEU2* promot-

er-*ATRP1m,* and *Eco*47III, *Spe*I, *Sac*II, *Sal*I, *Apa*I multicloning restriction sites for insertion) is flanked by 25S rDNA target sequences.

Xplor2.2-TEF1-2AUOX, which carries two AUOX expression modules in same orientation, was obtained by inserting the *TEF1* promoter – *AUOX* gene – *PHO5* terminator module flanked by *Spe*I restriction sites into Xplor2.2-TEF1-AUOX. The *TEF1* promoter – *AUOX* gene – *PHO5* terminator expression module was amplified by PCR from the *TEF1* promoter – *AUOX* gene – *PHO5* terminator fragment of plasmid pBS-TEF1-AUOX-PHO5-SS, using primers that incorporated the flanking *Spe*I cleavage sites (TEF1-SpeI/PHO5-SpeI; table 5).

In addition, a 6 × HisTag coding sequence was inserted at the 3′-end and at the 5′-end (behind the ATG) of the *AUOX* ORF using PCR. The complete gene construct, including the flanking restriction sites, was amplified by PCR from chromosomal DNA of *A. adeninivorans* LS3 using primer combinations AUOX5/AUOX3-His or AUOX5-His/AUOX3 (table 5) to construct genes *AUOX-6H* and *6H-AUOX*, respectively.

The amplified *Eco*RI-*Not*I *AUOX-6H* and *6H-AUOX* ORFs were inserted into plasmid pBS-TEF1-PHO5-SS as described before. Both of the resulting 6H-AUOX and AUOX-6H expression modules flanked by *Spe*I-*Sac*II restriction sites were inserted into Xplor2.2 to generate Xplor2.2-TEF1-AUOX-6H and Xplor2.2-TEF1-6H-AUOX.

All plasmids were digested with *Asc*I or *Sbf*I to remove all *E. coli* sequences prior to transformation. In the resulting cassettes, the module combination was flanked (YRCs) or not (YICs) by the two 25S rDNA fragments. All YRCs and YICs were introduced in linear form (fig. 2) into *A. adeninivorans* G1212.

Transformation Procedures, Isolation and Characterization of Nucleic Acids

Transformation of *E. coli* XL1-Blue competent cells from Stratagene followed the procedure described by Inoue et al. [1990]. *A. adeninivorans* transformants were generated according to Rösel and Kunze [1998]. Stable yeast transformants were obtained by a method described by Klabunde et al. [2003] after a sequence of passages on selective (YMM-glucose) and non-selective (YEPD) media. Isolation of plasmid DNA and restriction fragments was carried out as previously described [Wartmann et al., 2002].

For RNA isolation, yeast cells were suspended in lysis buffer (8 M guanidine hydrochloride, 20 mM MES, 20 mM EDTA, 1.25% (v/v) β-mercaptoethanol, pH 7.0). The isolation of total RNA was carried out according to Logemann et al. [1987]. To obtain DNA-free RNA, a DNase I (Fermentas, St. Leon-Rot, Germany) digestion step, as described in the manufacturer's instructions was included. cDNA was synthesized following the recommendations provided in the instruction manual '*Revert AidH Minus – First Strand cDNA Synthesis*' (Fermentas). In order to quantify the *AUOX* transcript accumulation by qRT-PCR, appropriate fragments were amplified by PCR from cDNA using the oligonucleotide primers listed in table 5. The relative expression level of *AUOX* mRNA was calculated as described by Hellemans et al. [2007] and Pfaffl [2001]. The *Arxula* genes *AHBS4*, *ALG9*, *TEF1* and *TFIID* were used as endogenous references (table 5).

Urate Oxidase Assays and Protein Analysis

Yeast cells were harvested (5,000 *g*, 4°C for 5 min), washed and suspended in 500 µl 0.1 M potassium phosphate buffer pH 8.0 and 500 µl silica beads (0.5 mm; BioSpec Products, Bartlesville, Okla., USA). The mixture was shaken 3 min at 30 Hz in Mill MM 400 mixer (Retsch, Haan, Germany), glass beads and cell material separated (11,000 *g*, 4°C, 10 min) and the supernatant used for the analyses.

Urate oxidase activity was assayed by quantification of (A) uric acid and (B) H_2O_2. For quantification A, the enzyme activity was determined spectrophotometrically by monitoring the decrease of absorbance at 293 nm using an extinction coefficient of 12.2×10^3 l/mol cm [Kalckar, 1947]. Standard assay mixtures, containing the sample, 0.1 M potassium phosphate buffer pH 8.0 and 0.2 mM uric acid, were incubated at 37°C. One unit of urate oxidase activity was defined as the ability to catalyze the transformation of 1 µmol uric acid to allantoin per minute at 37°C and pH 8.0.

For H_2O_2 quantification B, the urate oxidase activity was assayed by formation of red chromogen, which is produced by reaction of H_2O_2 with phenol, 4-aminoantipyrine and peroxidase as described by Lotfy [2008]. One unit of urate oxidase activity was defined as the amount of enzyme required to produce 1 µmol H_2O_2 per minute at 37°C and pH 8.0. Urate oxidase levels were expressed as U/mg protein or as U/l culture.

For the isolation and purification of the urate oxidase, *A. adeninivorans* cells were grown in YMM-glucose supplemented with 5 mM adenine (wild-type LS3) or 43.5 mM $NaNO_3$ (transformant) as nitrogen source. Cells were harvested, suspended in 0.1 M potassium phosphate buffer pH 8.0 and disrupted using a Mill MM 400 mixer (Retsch) with glass beads at 30 Hz. The proteins of the cell extract were fractionally precipitated using 35 and 65% ammonium sulfate (final concentration), centrifuged (4,000 *g*, 4°C, 15 min), dissolved in 0.1 M potassium phosphate buffer pH 7.4 and dialyzed against 50 mM potassium phosphate buffer pH 7.4 for 12 h.

The endogenous urate oxidase was subsequently purified to homogeneity by DEAE-Sepharose column chromatography and gel filtration on a Superdex™ 200 column [Böer et al., 2009a].

Recombinant urate oxidase (Auox 6h p), with a HisTag, was purified by column chromatography on Ni-NTA His breach Bind Resin (Novagen, Madison, Wisc., USA) and was desalted on PD10 desalting columns (Amersham Bioscience, Uppsala, Sweden) following the recommendations provided in the instruction manual.

The molecular masses of the enzymes purified from the wild-type and recombinant sources were estimated by gel filtration over Superdex™ 200. As calibration standards, dextran blue (M_r 2,000 kDa), ferritin (M_r 443 kDa), catalase (M_r 232 kDa), alcohol dehydrogenase (M_r 150 kDa), BSA (M_r 67 kDa) and ovalbumin (M_r 42.8 kDa) were used.

For further analysis, the endogenous urate oxidase Auoxp and recombinant Auox6hp were electrophoretically separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions [Laemmli, 1970]. Western analyses were performed as described by Towbin et al. [1979] and treated with anti-HisTag-specific antibodies as described by Böer et al. [2009a]. The dye-binding method of Bradford [1976] was used for protein quantification using bovine serum albumin (BSA) as standard.

Cells recovered from 1-ml samples of culture were washed twice in distilled water and dried until no further change in weight was noticed to determine the dry cell weight (dcw).

HPLC Analysis of Urate Oxidase Substrates and Products

To identify urate oxidase reaction products, the following compounds were tested: uric acid, guanine, xanthine and hypoxan-

thine (Sigma-Aldrich, St. Louis, Mo., USA) and adenine (Fluka Analytical, St. Gallen, Switzerland). Additionally, 0.2 U/ml urate oxidase was mixed with beef stock (REWE Bio Rinderbrühe; REWE, Germany, 1 g diluted in 1.5 ml 0.5 M NaOH + 18.5 ml 0.1 M potassium phosphate buffer pH 3.0) and incubated at 40°C. The reaction was stopped by heating at 100°C for 5 min. After centrifugation, aliquots (20 µl) of the supernatant were loaded onto an HPLC system (Hitachi LaChrom Elite® L-2130; Merck, Darmstadt, Germany) equipped with a TSKgel Amide-80 analytical column (250 × 4.6 mm; Tosoh, Tokyo, Japan) and fractionated by isocratic elution at a flow rate of 1.2 ml/min. Signals were detected at 260 nm. The eluent was acetonitrile and 0.1% trifluoroacetic acid (85:15 v/v) over a period of 20 min at 22°C. Purines were identified by comparison of their retention times with that of the pure uric acid, guanine, xanthine, hypoxanthine and adenine.

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References

Alamillo JM, Cárdenas J, Pineda M: Purification and molecular properties of urate oxidase from *Chlamydomonas reinhardtii*. Biochim Biophys Acta 1991;1076:203–208.

Antonenkov VD, Panchenko LF: Organization of urate oxidase in peroxisomal nucleoids. FEBS Lett 1978;88:151–154.

Arima K, Nose K: Studies on bacterial urate:oxygen oxidoreductase. I. Purification and properties of the enzyme. Biochim Biophys Acta 1968;151:54–62.

Arima K, Oshima T, Kubota I, Nakamura N, Mizunaga T, Tho-e A: The nucleotide sequence of the yeast *PHO5* gene: a putative precursor of repressible acid phosphatase contains a signal peptide. Nucleic Acids Res 1983;11:1657–1672.

Böer E, Bode R, Mock HP, Piontek M, Kunze G: Atan1p – an extracellular tannase from the dimorphic yeast *Arxula adeninivorans*: molecular cloning of the *ATAN1* gene and characterization of the recombinant enzyme. Yeast 2009a;26:323–337.

Böer E, Breuer FS, Weniger M, Denter S, Piontek M, Kunze G: Large-scale production of tannase using the yeast *Arxula adeninivorans*. Appl Microbiol Biotechnol 2011;92:105–114.

Böer E, Mock HP, Bode R, Gellissen G, Kunze G: An extracellular lipase from the dimorphic yeast *Arxula adeninivorans*: molecular cloning of the *ALIP1* gene and characterization of the purified recombinant enzyme. Yeast 2005; 22:523–535.

Böer E, Piontek M, Kunze G: Xplor®2 – an optimized transformation/expression system for recombinant protein production in the yeast *Arxula adeninivorans*. Appl Microbiol Biotechnol 2009b;84:583–594.

Böer E, Schröter A, Bode R, Piontek M, Kunze G: Characterization and expression analysis of a gene cluster for nitrate assimilation from the yeast *Arxula adeninivorans*. Yeast 2009c;26: 83–93.

Bongaerts GPA, Uitzetter J, Brouns R, Vogels GD: Uricase of *Bacillus fastidiosus*. Properties and regulation of synthesis. Biochim Biophys Acta 1978;527:348–358.

Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254.

Burns CM, Wortmann RL: Gout therapeutics: new drugs for an old disease. Lancet 2011;377: 165–177.

Dalbeth N: Progress and controversies in treatment. Nat Rev Rheumatol 2011;7:77–78.

Franke W, Krieg L: Purinoxydierende Fermente aus Schimmelpilzen II. Mitteilung: Zur Kenntnis der *Aspergillus*-Uricase. Chem Ber 1952;86:779–804.

Gienow U, Kunze G, Schauer F, Bode R, Hofemeister J: The yeast genus *Trichosporon* spec. LS3; molecular characterization of genomic complexity. Zentralbl Mikrobiol 1990;145:3–12.

Giersberg M, Degelmann A, Bode R, Piontek M, Kunze G: Production of a thermostable alcohol dehydrogenase from *Rhodococcus ruber* in three different yeast species using the Xplor®2 transformation/expression platform. J Ind Microbiol Biotechnol 2012;39: 1385–1396.

Giffard M, Ferté N, Ragot F, El Hajji M, Castro B, Bonneté F: Urate oxidase purification by salting-in crystallization: towards an alternative to chromatography. PLoS One 2011;5:e19013.

Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J: qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol 2007; 8:RI9.1–RI9.14.

Inoue H, Nojima H, Okayama H: High-efficiency transformation of *Escherichia coli* with plasmids. Gene 1990;96:23–28.

Kahn K, Serfozo P, Tipton PA: Identification of the true product of the urate oxidase reaction. J Am Chem Soc 1997;119:5435–5442.

Kai L, Ma XH, Zhou XL, Jia XM, Li X, Guo KP: Purification and characterization of a thermostable uricase from *Microbacterium* sp. strain ZZJ4-1. World J Microbiol Biotechnol 2008;24:401–406.

Kalckar HM: Differential spectrophotometry of purine compounds by means of specific enzymes. I. Determination of hydroxypurine compounds. J Biol Chem 1947;167:429–443.

Keebaugh AC, Thomas JW: The evolutionary fate of the genes encoding the purine catabolic enzymes in hominoids, birds, and reptiles. Mol Biol Evol 2010;27:1359–1369.

Keilin J: The biological significance of uric acid and guanine excretion. Biol Rev 1959;34:265–296.

Klabunde J, Kunze G, Gellissen G, Hollenberg CP: Integration of heterologous genes in several yeast species using vectors containing a *Hansenula polymorpha*-derived rDNA-targeting element. FEMS Yeast Res 2003;4:185–193.

Kurtzmann CP, Robnett CJ: Multigene phylogenetic analysis of the *Trichomonascus*, *Wickerhammiella* and *Zygoascus* yeast clades, and the proposal of *Sugiyamaella* gen. nov. and 14 new species combinations. FEMS Yeast Res 2007;7:141–151.

Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–685.

Leinmüller R: Der Hyperurikämie mit Fasturtec begegnen. Dtsch Arztebl 2001;98:3222.

Li J, Chen Z, Hou L, Fan H, Weng S, Xu C, Ren J, Li B, Chen W: High-level expression, purification, and characterization of non-tagged *Aspergillus flavus* urate oxidase in *Escherichia coli*. Protein Expr Purif 2006;49:55–59.

Liu J, Li G, Liu H, Zhou X: Purification and properties of uricase from *Candida* sp. and its application in uric acid analysis in serum. Appl Biochem Biotechnol 1994;47:57–63.

Liu X, Wen M, Li J, Zhai F, Ruan J, Zhang L, Li S: High-yield expression, purification, characterization, and structure determination of tag-free *Candida utilis* uricase. Appl Microbiol Biotechnol 2011;92:529–537.

Logemann J, Schell J, Willmitzer L: Improved method for the isolation of RNA from plant tissues. Anal Biochem 1987;163:16–20.

Lotfy WA: Production of a thermostable uricase by a novel *Bacillus thermocatenulatus* strain. Bioresour Technol 2008;99:699–702.

Lucas K, Boland MJ, Schubert KR: Uricase from soybean root nodules: purification, properties, and comparison with the enzyme from cowpea. Arch Biochem Biophys 1983;226: 190–197.

Middelhoven WJ, Hoogkamer-Te Niet MC, Kreger-Van Rij NJW: *Trichosporon adeninovorans* sp. nov., a yeast species utilizing adenine, xanthine, uric acid, putrescine and primary *n*-alkylamines as the sole source of carbon, nitrogen and energy. Antonie van Leeuwenhoek 1984;50:369–378.

Montalbini P, Redondo J, Caballero, JL, Cárdenas J, Pineda M: Uricase from leaves: its purification and characterization from three different higher plants. Planta 1997;202:277–283.

Nishimura H, Yoshida K, Yokota Y, Matsushima A, Inada Y: Physicochemical properties and states of sulfhydryl groups of uricase from *Candida utilis*. J Biochem 1982;91:41–48.

Oda M, Satta Y, Takenaka O, Takahata N: Loss of urate oxidase activity in hominoids and its evolutionary implications. Mol Biol Evol 2002;19:640–653.

Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:2002–2007.

Pfrimer P, de Moraes LMP, Galdino AS, Salles LP, Reis VCB, de Marco JL, Prates MV, Bloch C, Torres FAG: Cloning, purification, and partial characterization of *Bacillus subtilis* urate oxidase expressed in *Escherichia coli*. J Biomed Biotechnol 2010;2010:1–6.

Rajoka MI, Rehman KU, Meraj M, Akhtar MW, Zia MA: Purification, and properties of a bovine uricase. Protein Pept Lett 2006;13:363–368.

Rösel H, Kunze G: Integrative transformation of the dimorphic yeast *Arxula adeninivorans* LS3 based on hygromycin B resistance. Curr Genet 1998;33:157–163.

Scazzocchio C, Darlington AJ: The induction and repression of the enzymes of purine breakdown in *Aspergillus nidulans*. Biochim Biophys Acta 1968;166:557–568.

Schlesinger N, Yasothan U, Kirkpatrick P: Pegloticase. Nat Rev Drug Discov 2011;10:17–18.

Sorbera LA, Sharanya S, Dulsat C, Rosa E: Therapeutic targets for gout. Drug Future 2010;35: 333–337.

Steinborn G, Wartmann T, Gellissen G, Kunze G: Construction of an *Arxula adeninivorans* host-vector system based on *trp1* complementation. J Biotechnol 2007;127:392–401.

Suzuki K, Sakasegawa S, Misaki H, Sugiyama M: Molecular cloning and expression of uricase gene from *Arthrobacter globiformis* in *Escherichia coli* and characterization of the gene product. J Biosci Bioeng 2004;98:153–158.

Tanaka A, Ohishi N, Fukui S: Studies on the formation of vitamins and their function in hydrocarbon fermentation. IV. Production of vitamin B_6 by *Candida albicans* in a hydrocarbon medium. J Ferment Technol 1967;45: 617–623.

Terkeltaub R: Update on gout: new therapeutic strategies and options. Nat Rev Rheumatol 2010;6:30–38.

Towbin H, Staehelin H, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979;76:4350–4354.

Van der Walt JP, Smith MT, Yamada Y: *Arxula* gen. nov. (Candidaceae), a new anamorphic, arthroconidial yeast genus. Antonie van Leeuwenhoek 1990;57:59–61.

Votchitseva YA, Efremenko EN, Aliev TK, Varfolomeyev SD: Properties of hexahistidine-tagged organophosphate hydrolase. Biochemistry (Mosc) 2006;71:167–172.

Wang L-Y, Shih L-Y, Chang H, Jou S-T, Lin K-H, Yeh T-C, Lin S-F, Liang D-C: Recombinant urate oxidase (rasburicase) for the prevention and treatment of tumor lysis syndrome in patients with hematologic malignancies. Acta Haematol 2006;115:35–38.

Wartmann T, Krüger A, Adler K, Duc BM, Kunze I, Kunze G: Temperature-dependent dimorphism of the yeast *Arxula adeninivorans* LS3. Antonie van Leeuwenhoek 1995;68:215–223.

Wartmann T, Böer E, Pico AH, Sieber H, Bartelsen O, Gellissen G, Kunze G: High-level production and secretion of recombinant proteins by the dimorphic yeast *Arxula adeninivorans*. FEMS Yeast Res 2002;2:363–369.

Wittköpper K, Emons J, El-Armouche A: Febuxostat. Dtsch Med Wochenschr 2011;136:1270–1274.

Wolfram G: Das moderne Konzept der Ernährung bei Gicht. Akt Ernährungsmed 1992;17:24–32.

Wortmann RL: Gout and hyperuricamia. Curr Opin Rheumatol 2002;14:281–286.

Wu X, Muzny DM, Lee CC, Caskey CT: Two independent mutational events in the loss of urate oxidase during hominoid evolution. J Mol Evol 1992;34:78–84.

Yang XX, Wartmann T, Stoltenburg R, Kunze G: Halotolerance of the yeast *Arxula adeninivorans* LS3. Antonie van Leeuwenhoek 2000;77: 303–311.

Zhao Y, Yang X, Lu W, Liao H, Liao F: Uricase based methods for determination of uric acid in serum. Microchim Acta 2009;164:1–6.