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Production of orotic acid by a *Klura3*Δ mutant of *Kluyveromyces lactis*

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We demonstrated that a *Klura3*Δ, mutant of the yeast *Kluyveromyces lactis* is able to produce and secrete into the growth medium considerable amounts of orotic acid. Using yeast extract–peptone–glucose (YPD) based media we optimized production conditions in flask and bioreactor cultures. With cells grown in YPD 5% glucose medium, the best production in flask was obtained with a 1:12.5 ratio for flask: culture volume, 180 rpm, 28°C and 200 mM MOPS for pH stabilization at neutral values (initial culture pH at 8.0). The best production in a 2 L bioreactor was achieved at 500 rpm with 1 vvm aeration, 28°C and pH 7.0. Under these optimum conditions, similar rates of orotic acid production were obtained and maximum concentration achieved after 96 h was 6.7 g/L in flask and bioreactor cultures. These results revealed an excellent reproducibility between both systems and provided evidence for the biotechnological potential of *Klura3*Δ strain to produce orotic acid since the amounts obtained are comparable to the production in flask using a similar mutant of the industrially valuable *Corynebacterium glutamicum*.

[**Key words:** Bioprocess optimization; *Kluyveromyces lactis*; *KlURA3*; Orotic acid; Yeast biotechnology]

Kluyveromyces lactis is amongst the most studied non-conventional yeasts and has been increasingly used in both fundamental research and biotechnology industry (1,2). In this study we demonstrate that the pyrimidine-requiring mutant of *K. lactis* lacking the *KlURA3* gene is able to produce and secrete into the culture medium considerable amounts of a key intermediate from the *de novo* pyrimidines biosynthetic pathway – orotic acid (Fig. 1). Orotate salts, such as magnesium, calcium or lithium orotate, have been widely explored in the pharmaceutical and nutraceutical field. In the chemical and biotechnological industry, orotic acid has been used as starting material to produce pyrimidines and several other related molecules with higher market value (3–5). To our knowledge, a hypoxanthine-requiring *Candida tropicalis* strain and a pyrimidine-requiring *Candida albicans* strain were the only yeasts reported to accumulate orotic acid in culture media (6,7). *C. albicans Caura3Δ* mutants are able to accumulate orotic acid in yeast extract–peptone–glucose (YPD) media only if supplemented with acetate and *Saccharomyces cerevisiae ura3*Δ mutants do not present evidences for orotic acid accumulation in either media (7). Beside the presence of a transport system that facilitates secretion of orotic acid these evidences also suggest that specific metabolic and/or regulatory features may play a crucial role in determining if a particular microorganisms with similar mutation will be able to produce large amounts of orotic acid.

Studies on pyrimidines synthesis in *K. lactis* are scarce but it is well characterized in *S. cerevisiae*. Analysis of both genomes indicates a major difference in the step leading to the formation of orotic acid, which is the single redox reaction in the *de novo* pyrimidines biosynthesis pathway and is catalyzed by dihydroorotate dehydrogenases (DHODases) (Fig. 1). *S. cerevisiae* has only one gene for a DHODase (*URA1*) but the presence in *K. lactis* of two genes (*KlURA1* and *KlURA9*) coding for DHODases that belong to two distinct families is rather unexpected (8). Similar to its *S. cerevisiae* homologue, KlUra1p seems to belong to the DODHase family 1A, which includes cytosolic enzymes that use fumarate as electron acceptor. On the other hand, KlUra9p seems to belong to family 2, which includes enzymes that are localized in the inner mitochondrial membrane and deliver electrons to quinone (8,9). Although no functional or localization studies have been made in *K. lactis*, this suggests that its *de novo* pyrimidine biosynthesis pathway may be coupled to the mitochondrial respiratory chain via the KlUra9p DHODase.

Whether it is this particular feature of the *de novo* pyrimidines biosynthetic pathway or other distinctive metabolic characteristic of *K. lactis* that account for the amount of orotic acid produced by the *Klura3*Δ mutant, remains to be clarified. In this study, we explored and evaluated the potential of *K. lactis Klura3*Δ as an orotic acid producing microorganism. Growth conditions and culture media based on YPD were optimized for orotic acid production in both flask and bioreactor.

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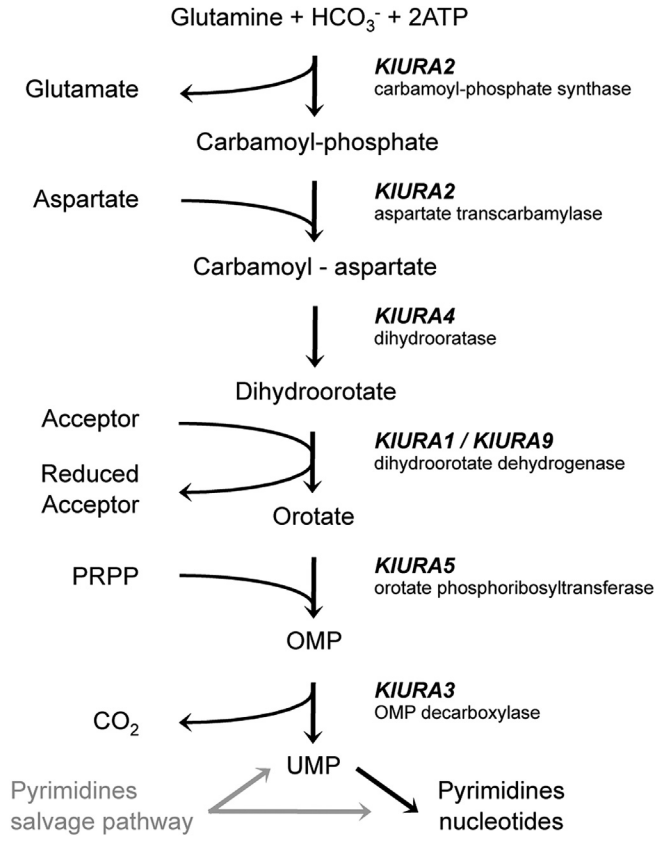

FIG. 1. Pyrimidine biosynthetic pathway. Six enzymatic steps lead to the formation of uridine-5′-phosphate (UMP) in *de novo* pyrimidines biosynthesis from which all pyrimidines nucleotides are further synthesized (black). Pyrimidines salvage pathway rely on a set of reactions in order to reutilize pyrimidines nucleosides and bases, either intracellular or internalized from the culture media, which can enter the pathway directly at UMP or at later steps (gray). OMP, orotidine-5′-phosphate.

MATERIALS AND METHODS

***K. lactis* strains** *Klura3*Δ and *Klura5*Δ mutants were generated from *K. lactis* DSM 70799 wild type strain (DSMZ, Braunschweig, Germany). *Klura3*Δ was used to generate *Klura1*Δ*Klura3*Δ, *Klura9*Δ*Klura3*Δ and *Klura1*Δ*Klura9*Δ*Klura3*Δ mutants. Full length of the open reading frame (ORF) of *KlURA3*, *KlURA5*, *KlURA1* and *KlURA9* genes were removed to generate the various deletion mutants. All primers used in this study (A1/A2 and B1/B2 sets for each gene) are mentioned in Table 1. Each ORF with flanking regions were amplified by PCR from *K. lactis* DSM 70799 genomic DNA using A1/A2 set of primers and then cloned into pGEM-T Easy (Promega, Madison, WI, USA). A fragment of this construct containing only the flanking regions of each ORF was amplified by PCR using the B1/B2 set of primers and a *Pvu*II/*Eco*RV fragment containing *loxP*–*kanMX4*–*loxP* from pUG6 (Euroscarf, Frankfurt, Germany) was ligated into the open vector. Each deletion cassette was then amplified with A1/A2 primers and used to transform the parental strain by electroporation. The mutants were selected in YPD [1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose] supplemented with geneticin (200 mg/L) and correct deletion was confirmed by PCR using a set of primers 400 bp upstream A1/A2. Cre/loxP-mediated marker removal procedure (10) was used to excise *KanMX4*.

TABLE 1. Primers used in this study.

Primer	Sequence (5′- 3′)
A1-Ura3	CACTGTCTCTTCCCCTTAATGA
A2-Ura3	TGTGTGCTTGCTTCTTTTCTTATC
B1-Ura3	GTGCAACTAATTGACGGGAGT
B2-Ura3	CAGGAAACTTAATAGAACAAATCACA
A1-Ura5	GCCTTATCAGGATCAGATGAAG
A2-Ura5	CAAAGACACATCCACAAGATTTTG
B1-Ura5	TGAACAGGTGATTAATGGCGGA
B2-Ura5	TATCACCCTCGAATCTATCTTAAC
A1-Ura1	CCGAATAGTATCTGTGACTAAGA
A2-Ura1	CTACTTCTTCAGTATTAGCCTTTC
B1-Ura1	TGTTCAGTCTTACCTTGAATGTTTAT
B2-Ura1	TTTTTCATATAGCGGTGTTTAGTATAT
A1-Ura9	AATATTGATTCGGCTTCTCGTGA
A2-Ura9	TCCAACAGAATCCCAAACCCA
B1-Ura9	AGTTGATAAAAGCAAAATACGCGG
B2-Ura9	GCTGTTACCTAATTGAAGTAAGG

Media, inoculation and growth The growth media used were YPD and YPD 5% glucose [1% (w/v) yeast extract, 2% (w/v) bactopeptone, 5% (w/v) glucose]. The set-up for inoculation of all cultures was as follows: (i) 4°C stock culture: a YPD culture grown to 2.5 g/L d.w. was centrifuged at 3000 g for 5 min and resuspended in 1/10 of the same culture medium, stored at 4°C and used for preparing every pre-culture; this stock culture was substituted every month but could be used longer without any effect in the pre-culture growth behavior or orotic acid production; (ii) pre-culture: an appropriate volume from the 4°C stock culture (20 µl per ml pre-culture) was used to inoculate YPD medium and cells were grown for 24 h at 26°C and 180 rpm to a biomass concentration of 4.0 g/L d.w.; (iii) culture: irrespective of the growth parameter tested, culture medium composition and flask or bioreactor experiments, the equivalent to 1/10 of culture medium volume from the pre-culture was used for inoculation.

Several growth conditions were tested in flask and bioreactor and are described in the results section and figure legends. Potassium phthalate and MOPS were obtained from Sigma–Aldrich. Bioreactor experiments were performed in a Biostat B2 (Sartorius, Goettingen, Germany) equipped with 405-DPAS-SC-K8S/200 pH probe (Mettler-Toledo, Columbus, OH, USA) and InPro 6800 pO_2 sensor (Mettler-Toledo). Solutions used for pH correction were 2 M NaOH and 2 M HCl. Air supply was controlled by an MC-10SLPM mass flow controller (Alicat Scientific, Tucson, AZ, USA). Aeration, agitation, temperature and pH were automatically controlled and maintained constant for every culture.

Crystals characterization Diffraction data from X-ray crystallography were collected at 293 K with a Gemini PX Ultra equipped with CuK_α radiation ($\lambda = 1.54184$ Å). The structure was solved by direct methods using SHELXS-97 and refined with SHELXL-97 (11). Carbon, oxygen, nitrogen and potassium atoms were refined anisotropically. Hydrogen atoms were refined freely with isotropic displacement parameters. The refinement converged to R (all data) = 5.55% and wR_2 (all data) = 13.09%.

The scanning electron microscopy with X-ray microanalysis (SEM/EDS) exam was performed using a high resolution scanning electron microscope with X-Ray Microanalysis, JEOL JSM 6301F/Oxford INCA Energy 350. Samples were coated with a Au/Pd thin film, by sputtering, using the SPI Module Sputter Coater equipment.

HPLC quantification of orotic acid and glucose For orotic acid quantification, an Hitachi HPLC system was used and consisted of the following components: L-7100 pump, L-7250 autosampler, PCR7250 peltier cooling rack, L-7300 Plus column oven, L-7400 UV detector and D-7000 interface module. The column was a XBridge C_{18} (3.5 µm; 4.6 mm × 150 mm) from Waters (Milford, MA, USA) and was kept at 22°C. Mobile phase consisted of 25 mM K_2HPO_4/KH_2PO_4 at pH 7.0 and flow rate was 0.5 ml/min for a total run time of 40 min. Column effluent was monitored at 280 nm. Orotic acid standard was obtained from Sigma–Aldrich.

Glucose concentrations were determined by HPLC in a Jasco chromatograph equipped with refractive index (RI) detector (Jasco 830-RI) and a Chrompack (300 mm × 6.5 mm) column at 60°C, using 5 mM H_2SO_4 as eluent at a flow rate of 0.5 ml/min.

RESULTS AND DISCUSSION

***K. lactis Klura3*Δ mutant as an orotic acid producing microorganism** YPD agar plates with fully grown *Klura3*Δ mutant cells of *K. lactis* started to display two types of crystal structures after about 1 week at 4°C. One type was rather large presenting a well defined star-like structure (Fig. 2A) while the other crystals were smaller and abundant (Fig. 2B). X-ray crystallography analysis of both type of crystals demonstrated that its constituent matched the structure of orotate, which was present as a monohydrated salt (Fig. 2C and D). Orotate counter-ion was identified as potassium by SEM/EDS examination (Fig. S1). The presence of two morphological structures is likely a result of distinct crystal nucleation and growth when kept at 4°C. For the matter of simplicity, despite these crystals were composed of potassium orotate monohydrate, the molecule produced by this mutant, either in crystal structures or dissolved, will be further referred as orotic acid.

We did not observe this phenotype in the wild type strain or in the triple mutant *Klura1*Δ*Klura9*Δ*Klura3*Δ, in which the step prior