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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*.

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[**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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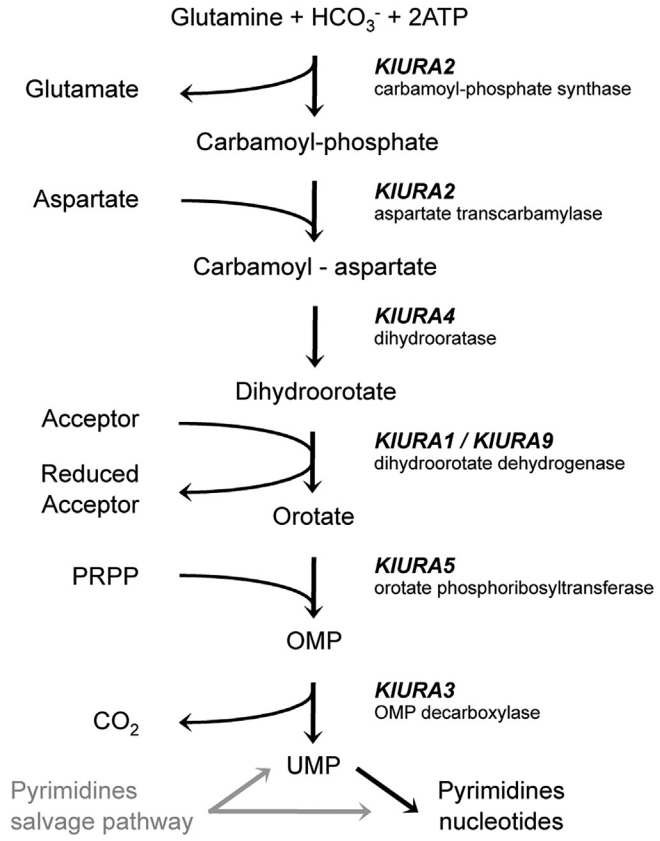

FIG. 1. Pyrimidine biosynthetic pathway. Six enzymatic steps lead to the formation of uridine-5′-phosphate (UMP) in *de novo* pyrimidines biosynthesis from which all pyrimidines nucleotides are further synthesized (black). Pyrimidines salvage pathway rely on a set of reactions in order to reutilize pyrimidines 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

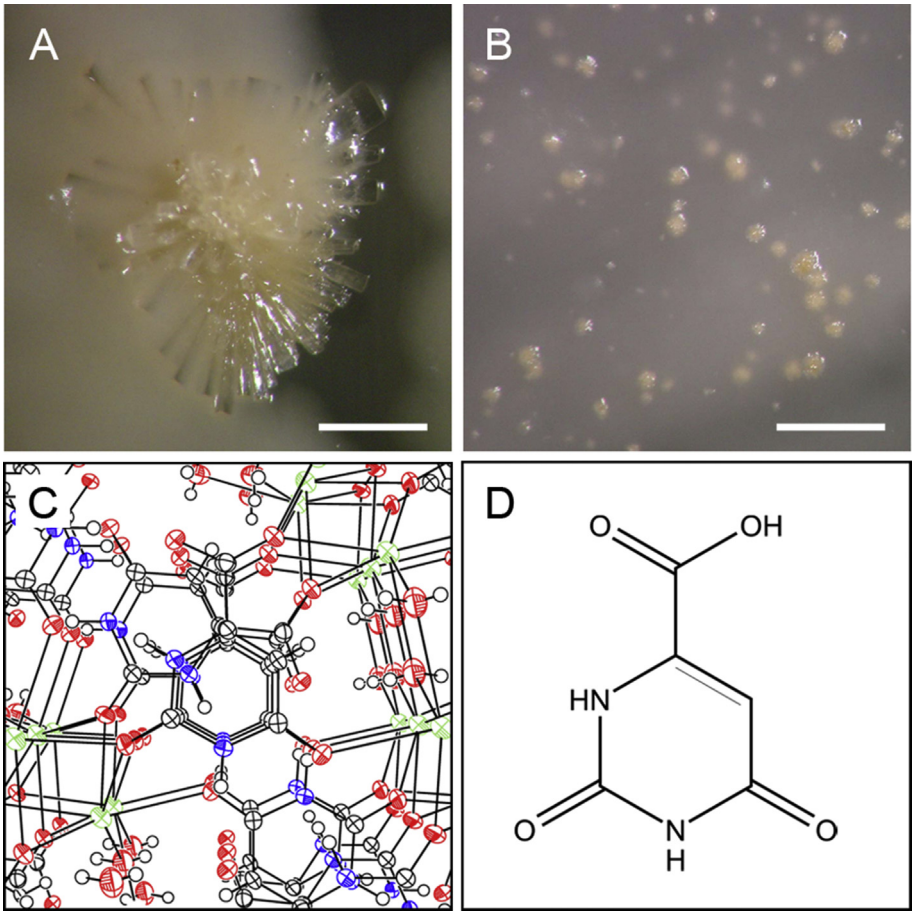

FIG. 2. Large (A) and small (B) crystal structures. Scale bars represent 1 mm. (C) Crystal network of potassium orotate monohydrate determined by x-ray crystallography. In the center of the figure is a set of overlapping orotate molecules in the same disposition as the molecular structure depicted in Fig. 2D. K in green, O in red, N in blue, C in black and H in open circles. (D) Molecular structure of orotic acid. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to the formation of orotic acid is interrupted along with *KlURA3* deletion (Fig. 1). Also, orotic acid was not detected in the medium of wild type and *Klura1*Δ*Klura9*Δ*Klura3*Δ cells grown in liquid cultures (data not shown), in contrast to what was observed with *Klura3*Δ mutants (see below). These evidences demonstrated that the interruption of *de novo* pyrimidines biosynthetic pathway by deleting *KlURA3* led to the accumulation of orotic acid generated by the irreversible oxidative reaction catalyzed by the DHODases (KlUra1p and KlUra9p). Due to the existence of an intermediate step catalized by orotate phosphoribosyltransferase (KlURA5p) (Fig. 1) the mutant *Klura5*Δ was also generated and tested. This mutant exhibited similar phenotype to the observed in *Klura3*Δ cells, producing equivalent amounts of orotic acid in liquid cultures under any conditions tested. Therefore, for simplicity, only results obtained with *Klura3*Δ mutants are presented. Similar orotic acid production in *Klura3*Δ and *Klura5*Δ mutants is most likely due to a limiting phosphoribosyl pyrophosphate (PRPP) availability (Fig. 1) that, together with the reversible nature of the enzymatic step catalyzed by KlUra5p, favors orotic acid accumulation upon *KlURA3* deletion.

Production of orotic acid in flask cultures To evaluate orotic acid production yields, *Klura3Δ* mutant was grown in flask cultures and several growth parameters were tested. Oxygen requirements of *Klura3*Δ mutant and its interference in orotic acid production were tested in different culture: flask volume ratio conditions using 250 ml flat bottom flasks agitated at 180 rpm, providing this way different levels of culture aeration (Fig. 3A). Although the different ratios tested did not affect cell growth, major differences were observed in the rate of orotic acid production. Production rate was negatively affected below 1:8.3 ratio (30 ml:250 ml) and was maximal with a 1:12.5 ratio (20 ml:250 ml) that led to a concentration of about 2.2 g/L after 48 h. We also evaluated the production at higher aerations using 20 ml cultures in 250 ml baffled flasks agitated at 180 rpm and 250 rpm but no further improvement was observed (Fig. S2). Therefore, aeration conditions for further experiments were set to 20 ml culture in 250 ml flat bottom flasks agitated at 180 rpm. These results show that an additional oxygenation above the levels that already allow normal cell growth is required for optimal production rate. This is consistent with the presence of a putative mitochondrial Klura9p that links pyrimidines pathway to the respiratory chain and is most likely playing a major role in the enzymatic step leading to the formation of orotic acid, in contrast to the other DODHase KlUra1p. This hypothesis was confirmed by the analysis of orotic acid production in the double mutants *Klura1*Δ*Klura3*Δ and *Klura9*Δ*Klura3*Δ. These mutants exhibited similar growth rates but, whereas orotic acid production was similar using *Klura3*Δ and *Klura1*Δ*Klura3*Δ mutants, it was strongly and negatively affected using *Klura9*Δ*Klura3*Δ mutant (Fig. S3). This mutant was only able to produce 0.7 g/L of orotic acid after 48 h. Therefore, oxygen seems to be directly linked to orotic acid production due to the predominant or more efficient activity of KlUra9p DODHase.

Using the best conditions already defined, different growth temperatures from 24°C to 32°C were tested (Fig. 3B). Although cell

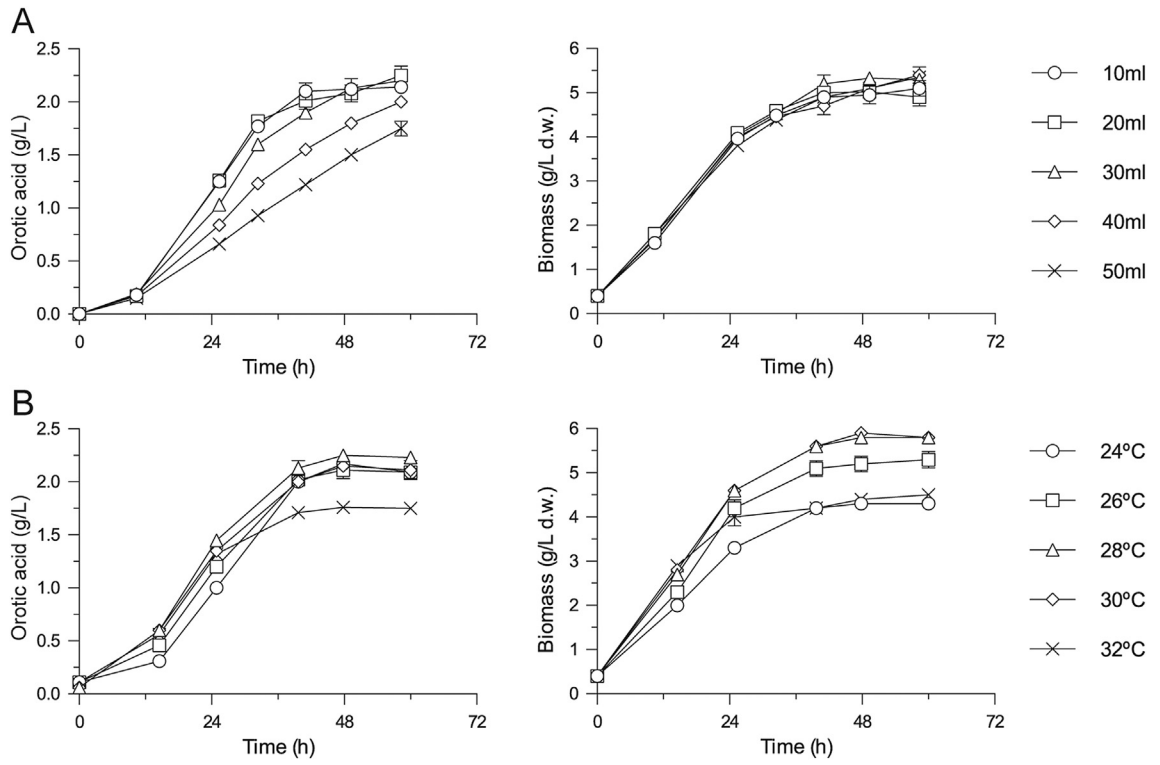

FIG. 3. Effect of aeration (A) and temperature (B) on orotic acid production and growth in flask cultures. (A) Increase in aeration was achieved by incrementally decreasing volumes of YPD growth medium (50 ml to 10 ml) in flat bottom 250 ml flasks agitated at 180 rpm; growth was performed at 26°C. (B) YPD cultures (20 ml) were grown at different temperatures (24°C to 32°C) in 250 ml flat bottom flasks agitated at 180 rpm. Data represent mean values and standard deviations of duplicate experiments.

growth was lower at 24°C and 32°C, orotic acid production at 24°C was only delayed during the early stage, while at 32°C the maximum amount of orotic acid produced was decreased. At 24°C specific productivity was therefore higher than at temperatures from 26°C to 30°C. The lower biomass and orotic acid production at 32°C is probably associated with temperature sensitivity of *Klura3*Δ mutant at a particular growth stage (after 12 h) or with deviation of energy or resources (glucose or other nutrients) to other mechanisms or metabolic pathways required for growth at this temperature. Since the best results were obtained at 26°C–30°C with minor differences in both growth and orotic acid production, 28°C was chosen as the growth temperature for further optimizations.

Under these best conditions, the initial pH (ipH) of the culture medium dropped from 6.6 (pH of YPD medium) to 4.4 during the first 36 h and then remained constant (Fig. S4). To evaluate the effects of pH in the production process different buffering systems were tested. For these experiments, glucose concentration was increased from the 20 g/L used in standard YPD to 50 g/L (YPD 5% glucose) nor only to show that the culture media could be improved and a glucose increment alone was sufficient to significantly increase orotic acid production but also because it would better demonstrate the efficacy of the buffering system under conditions of stronger acidification. Data regarding pH behavior of the cultures and its effect on orotic acid production and cell growth are summarized in Fig. 4. Cells grown in unbuffered YPD 5% glucose with an ipH 6.6 as in previous experiments, produced 2.9 g/L after 48 h and 3.9 g/L after 72 h and culture pH dropped from 6.6 to 3.4. Optimum pH for growth of *K. lactis* wild type strains is known to be 4.5–5.0 and for that reason phthalate (pK_a 5.5) was used to buffer the cultures and maintain pH values close to that range. The

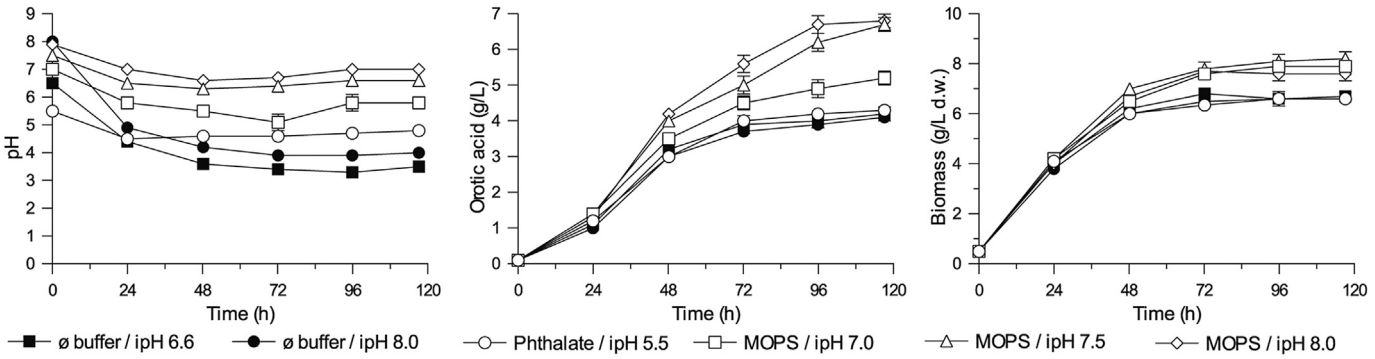

FIG. 4. pH behavior of unbuffered and buffered cultures and its effect on orotic acid production and growth in flask cultures. Phthalate (75 mM) at ipH 5.5 and 200 mM MOPS at ipH 7.0, 7.5 and 8.0 were used to stabilize culture pH above the values obtained with unbuffered cultures at ipH 6.6 and 8.0. Growth was performed using 20 ml YPD 5% glucose in 250 ml flat bottom flasks at 28°C and 180 rpm. Data represent mean values and standard deviations of duplicate experiments.

concentration of phthalate used (75 mM) did not affect the growth of *Klura3*Δ cells and was sufficient to prevent a drop over one pH value from an ipHs of 5.5. However, stabilizing pH with phthalate for most of the culture period at about 4.7 did not improve orotic acid production.

To test orotic acid production in cultures with pH stabilized at higher values, MOPS buffer (pK_a 7.2) was used. MOPS has lower buffering capacity than phthalate and a concentration of 200 mM was required to prevent a drop of pH higher than 1–1.5 in the cultures tested without having a negative effect on growth (Fig. 4). In fact, some increase in final biomass was observed in cultures buffered with MOPS. Under these conditions, orotic acid concentration far exceeded its solubility (1.8 g/L in water at 18°C) and microscopic spike like crystals were already visible at 48 h. Therefore, for orotic acid quantification, concentrated NaOH was added to a final concentration of 250 mM to dissolve orotic acid crystals in the samples (solubility in 1 M NaOH is 50 g/L). The results shown in Fig. 4 clearly show that stabilizing culture pH with MOPS at values closer to 7 strongly increased orotic acid production. Both unbuffered cultures with ipH 6.6 and ipH 8.0 acidified to pHs below 4. On the other hand, MOPS cultures that started at ipH 8.0 maintained its pH close to 7. Whereas unbuffered cultures only produced about 3.9 g/L after 72 h without significant increase up to 96 h, MOPS cultures with ipH 8.0 were able to produce 5.6 g/L after 72 h and 6.7 g/L after 96 h. Culture pH is therefore critical for orotic acid production and, due to the strong tendency of the culture to acidify, the use of this buffer system is essential to sustain pH at optimal values. It was not possible to test orotic acid production in buffered cultures with pH stabilized at even higher values, either with MOPS or other buffer with higher pK_a, since ipH of the culture would necessarily have to be increased and cell growth is affected above pH 8. Nevertheless, taking into account the differences observed between MOPS cultures at ipH 8.0 and ipH 7.5, a significant improvement in production would not be expected if pH could be stabilized above 7.0. Moreover, as shown in the following section, maintaining culture pH at 8.0 in a bioreactor had a negative

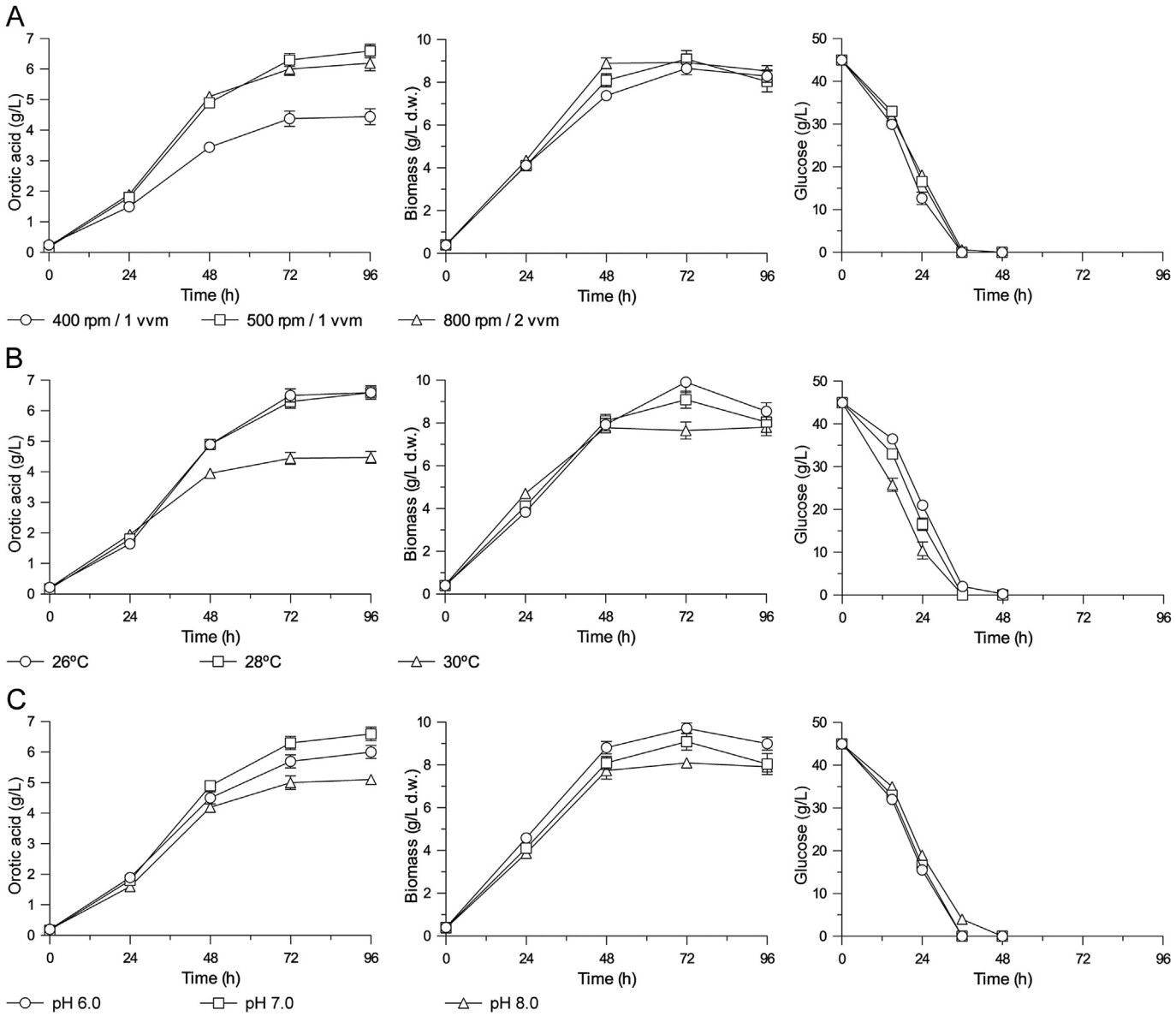

FIG. 5. Effect of agitation (rpm)/aeration (vvm) (A), temperature (B) and pH (C) on orotic acid production, growth and glucose consumption in bioreactor cultures. For each set of experiments the following conditions were maintained constant: (A) temperature and pH respectively at 28.0°C and pH 7.0; (B) agitation, aeration and pH respectively at 500 rpm, 1 vvm and pH 7.0; (C) agitation, aeration and temperature respectively at 500 rpm, 1 vvm and 28.0°C. Data represent mean values and standard deviations of duplicate experiments.

effect on the production. With respect to glucose concentration, we observed that the increase of orotic acid production was only relevant if glucose was increased up to 50 g/L. In a buffered culture at ipH 8.0, an additional 25 g/L of glucose (YPD 7.5% glucose) only generated a 0.6 g/L increment of orotic acid (Fig. S5). In conclusion, under the conditions tested, 200 mM MOPS in YPD 5% glucose with ipH 8.0 was considered as the most adequate buffering system for orotic acid production in flask cultures.

The results obtained from cultures buffered with phthalate and MOPS demonstrated that *Klura3*Δ cells can grow well at any pH from about 4.5 to 7 and that pH 7 is probably the most favorable for specific mechanisms involved in the production or secretion of orotic acid. Available data on orotic acid producing strains with industrial value (12) show that a similar mutant of *Corynebacterium glutamicum*, with pyrimidines pathway interrupted and no further genetic optimization, is able to produce in flask cultures 8.2 g/L of orotic acid after 120 h. Having reached an orotic acid concentration of 6.7 g/L at 96 h in flasks, our results demonstrated the high potential of *K. lactis Klura3*Δ as an orotic acid producing microorganism. Orotic acid production by *C. glutamicum* strains was obtained using optimized and cheap production media (12). Production by *K. lactis Klura3*Δ using YPD based media would not be used in industry due to its costs and full media optimization should be performed to determine the best combinatorial composition using general and specific cheap nutrients that should be previously identified as relevant for orotic acid production. Results already obtained in this work strongly suggest that with an optimized production media and an additional 24 h production time, final amounts of orotic acid above 8.2 g/L could be reached after 120 h using *K. lactis Klura3*Δ.

Production of orotic acid in bioreactor To fully assess the value of *K. lactis Klura3*Δ strain for the biotechnology industry, the production process was further tested in a bioreactor system. Using the information obtained from flask experiments and YPD 5% glucose as production medium (1 L), several conditions of agitation, aeration, pH and temperature were tested in a 2 L bioreactor (Fig. 5). As for flask cultures with MOPS buffer, NaOH was added to all samples removed from cultures after 24h to dissolve orotic acid crystals and allow its quantification by HPLC. All results obtained in flask cultures regarding optimum conditions for orotic acid production were confirmed in the bioreactor. First, with temperature and pH maintained constant at 28°C and pH 7.0, a minimum aeration/agitation (500 rpm/1 vvm) above the one required for optimal growth of this mutant strain, is essential and sufficient for best orotic acid production rate and yield (Fig. 5A). For each aeration conditions we observed that pO_2 dropped to about 80% (800 rpm/2 vvm), 40% (500 rpm/1 vvm) and 10% (400 rpm/1 vvm) (data not shown), thus indicating culture pO_2 should not drop below 40%. Second, at pH 7.0 and optimum aeration/agitation conditions, orotic acid production was higher at 26°C and 28°C, being significantly affected at 30°C (Fig. 5B). Third, at 28°C and optimum aeration/agitation conditions, best orotic acid production was obtained at neutral pH (Fig. 5C) indicating that optimum pH for orotic acid production would be very close or at pH 7.0. Regarding glucose consumption, despite a minor difference observed at 30°C (Fig. 5B), the results were similar for all other conditions tested (Fig. 5A and C). Strikingly, the *Klura3*Δ mutant strain was still able to accumulate in the growth media large amounts of orotic acid after glucose has been depleted. Also, differences in orotic acid production under the different conditions tested were mostly observed after that point. This suggests that steps of synthesis *de novo* leading to orotic acid formation may not be under an effective negative control in this mutant, leading to a continuous production under optimum conditions as long as substrates are available. Production in bioreactor was optimal with growth parameters set to 28°C, 500 rpm, 1 vvm and pH 7.0 and the amount of orotic acid obtained after 96 h was 6.7 g/L (Fig. 5A, B or C). Analyzing these results together with the production obtained in flasks under the optimized conditions already defined (MOPS/ipH 8.0, Fig. 4), it is evident that the rates of production are similar and the same maximum amount of orotic acid is reached at 96 h.

Production scale-up using *K. lactis* have been made and proven useful for the biotechnology industry. In this work, we demonstrated that the biotechnological potential of the *K. lactis Klura3*Δ as an orotic acid producing strain is highly relevant and scaling-up using further optimized strains and media may lead to an even more efficient and industrially competitive production process when compared to the *C. glutamicum* system. The high reproducibility of orotic acid production in flask and bioreactor indicates that the flask system is suitable to be used as standard for evaluating further genetic optimization on *Klura3*Δ strain prior to bioreactor tests. *K. lactis* is close related to the well studied yeast *S. cerevisiae*, its genome has been fully sequenced and many specific genetic tools have been developed. Identification of genetic targets and directed mutagenesis could be easily achieved and already assessed with this YPD/MOPS flask system. It could also be further and easily adapted for screening of random mutants in high-throughput systems. Moreover, flask experiments to elaborate the composition of a production and industrial medium made of cheap and more defined constituents are expected to be replicated in bioreactor at any stage of the development process.

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jbiosc.2015.10.008.

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