Production of orotic acid by a Klura3Δ mutant of Kluyveromyces lactis

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Received 24 June 2015; accepted 15 October 2015
Available online 18 December 2015

We demonstrated that a Klura3Δ, 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 1vvm 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 Klura3Δ 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 ura3Δ mutants do not present evidences for orotic acid accumulation in either media (7). Besides 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 (DHOases) (Fig. 1). S. cerevisiae has only one gene for a DHOase (URA1) but the presence in K. lactis of two genes (KURA1 and KlURA9) coding for DHOases 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 DHOase.

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 Klura3Δ mutant, remains to be clarified. In this study, we explored and evaluated the potential of K. lactis Klura3Δ 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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http://dx.doi.org/10.1016/j.jbiosc.2015.10.008
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 μg/mL) 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.

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].

RESULTS AND DISCUSSION

K. lactis Klura3Δ mutant as an orotic acid producing microorganism
YPD agar plates with fully grown Klura3Δ 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 Klura1ΔKlura9ΔKlura3Δ, in which the step prior
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 Klura1ΔKlura9ΔKlura3Δ cells grown in liquid cultures (data not shown), in contrast to what was observed with Klura3Δ 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 DODHases (KlUra1p and KlUra9p). Due to the existence of an intermediate step catalyzed by orotate phosphoribosyltransferase (KlURA5p) (Fig. 1) the mutant Klura5Δ was also generated and tested. This mutant exhibited similar phenotype to the observed in Klura3Δ cells, producing equivalent amounts of orotic acid in liquid cultures under any conditions tested. Therefore, for simplicity, only results obtained with Klura3Δ mutants are presented. Similar orotic acid production in Klura3Δ and Klura5Δ 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 Klura3Δ 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 aeration 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 Klura1ΔKlura3Δ and Klura9ΔKlura3Δ. These mutants exhibited similar growth rates but, whereas orotic acid production was similar using Klura3Δ and Klura1ΔKlura3Δ mutants, it was strongly and negatively affected using Klura9ΔKlura3Δ 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. 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.)**
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 KluraΔ 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) not 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 (pKa 5.5) was used to buffer the cultures and maintain pH values close to that range. The
concentration of phthalate used (75 mM) did not affect the growth of Klura3Δ cells and was sufficient to prevent a drop over one pH value from an ipH 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 (pKa 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 pKa, 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

![Graphs showing effects of agitation, aeration, temperature, and pH on orotic acid production, growth, and glucose consumption in bioreactor cultures.](image-url)
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 pH 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 pH 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 Klura3Δ 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 genetic optimization, is able to produce in 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 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 Klura3Δ 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 Klura3Δ.

Production of orotic acid in bioreactor To fully assess the value of K. lactis Klura3Δ 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 24 h 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 pO2 dropped to about 80% (500 rpm/2 vvm), 40% (500 rpm/1 vvm) and 10% (400 rpm/1 vvm) (data not shown), thus indicating culture pO2 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 Klura3Δ mutant strain was still able to accumulate in the growth medium 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/pH 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 Klura3Δ 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 Klura3Δ 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.

ACKNOWLEDGMENTS

This work was funded by FEDER funds through the Operational Competitiveness Programme — COMPETE and by National funds through FCT — Fundação para a Ciência e a Tecnologia under the project FCOMP-01-0124-FEDER-037277 (Pest-C/SAU/LA0002/2013). Nuno Carvalho was supported by a FCT fellowship (SFRH/BD/19571/2004).

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