

THE NADP⁺-DEPENDENT GLUTAMATE DEHYDROGENASE OF THE YEAST *KLUYVEROMYCES MARXIANUS* RESPONDS TO NITROGEN REPRESSION SIMILARLY TO *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

NADP⁺-dependent glutamate dehydrogenase (NADP⁺-Gdh) is the first step in ammonia assimilation pathway in *Saccharomyces cerevisiae* and the knowledge of its regulation is the key for many biotechnological purposes such as single cell protein production. The regulation of NADP⁺-Gdh activity in *Kluyveromyces marxianus* cells was evaluated under different ammonia supply in batch cultivations. The results showed that *K. marxianus* NADP⁺-Gdh activity is induced over a narrow range of extracellular ammonia supply, being repressed by both high ammonia concentration and the glutamate formed. This activity is not growth-associated and may function mainly to trace low amounts of ammonia after growth cessation. The results demonstrated that NADP⁺-Gdh may not be the main enzyme for ammonia assimilation in *K. marxianus*, as it has been postulated for *K. lactis*, instead is subjected to the same regulatory mechanism described for *S. cerevisiae*.

Key words: ammonia assimilation, enzyme activity, glutamate dehydrogenase, *Kluyveromyces marxianus*.

INTRODUCTION

The availability of intracellular glutamate is essential for the transamination reactions involved in the amino acids biosynthetic pathways. In *Saccharomyces cerevisiae* cells there are two routes for glutamate formation. The first is considered to be the main glutamate-producing pathway in this yeast. The ammonia uptake from the medium is used to produce glutamate by the amination of 2-oxoglutarate by NADP⁺-dependent glutamate dehydrogenase (E.C. 1.4.1.4) (NADP⁺-Gdh: 2-oxoglutarate + NADPH + NH₄⁺ → glutamate + NADP⁺), followed by the amination of glutamate to glutamine by glutamine synthase (E.C. 6.3.1.2) (Gs: glutamate + ATP → glutamine + ADP). In the second, glutamate is also produced in a transamination reaction, which transfers one amino group from glutamine to 2-oxoglutarate, catalysed by the glutamate synthase or glutamate-2-oxoglutarate aminotransferase (E.C. 1.1.1.13) (Gogat: glutamine + 2-oxoglutarate + NADH → 2 glutamate + NAD⁺) (9). When

extracellular ammonia concentration exceed 5 mM, *S. cerevisiae* cells use preferentially NADP⁺-Gdh, whereas Gs-Gogat pathway is activated at low ammonia concentrations. On the other hand, *Kluyveromyces lactis* cells seem to use the Gs-Gogat pathway as the main glutamate biosynthetic pathway (12,14). Although this ATP-consuming pathway is energetically unfavourable, this could be related to the maintenance of redox equilibrium to re-oxidise the excess of NADH produced during glycolysis, since ethanolic fermentation does not occur in this yeast under oxygen supply (8).

It has been related the biotechnological use of *Kluyveromyces* in the "Yeast as Cell Factory" concept to produce amino acid-rich biomass due to its advantageous physiological characteristics compared to *S. cerevisiae*: the absence of ethanol production (Crabtree-negative effect), the lower catabolic repression response, the higher flux through the glycolytic pathway (8,15) and the high capacity of the respiratory enzymes (6,15). The close-related specie *Kluyveromyces marxianus* has been used

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to produce bioingredients, such as oligopeptides, oligosaccharides and oligonucleotides mainly for pharmaceutical and food industries (1). Similarly to *K. lactis*, *K. marxianus* is Crabtree-negative yeast, generally regarded as safe for human and animal consumption and possesses an arsenal of hydrolytic extracellular hydrolases that enables its use for bioconversion of complex sugar-rich substrates into single cell protein. There are no reports in the literature regarding enzymes activities and its regulation on the nitrogen assimilation pathways in *K. marxianus*. Therefore, the necessity to understand those pathways precedes the efforts to produce amino acids-enriched *K. marxianus* biomass. The aim of this work was to study the behaviour of the NADP⁺-glutamate dehydrogenase activity of *K. marxianus* cells under different batch cultivation conditions. The results showed that the enzyme activity responds to the extracellular concentration of ammonia, being inhibited by high substrate concentrations. Moreover, it seemed to be controlled by the availability of ammonia or by the presence of free amino acids in the medium.

MATERIALS AND METHODS

Yeast strain and culture condition

The prototrophic and diploid homothallic *K. marxianus* CBS6556 type strain was used in this work. Cells were maintained freshly in YPD medium and one colony was used to inoculate 50 mL mineral medium containing 1.7 g.L⁻¹ yeast nitrogen base (w/o amino acids and ammonium sulfate), 20 g.L⁻¹ glucose and 5 g.L⁻¹ ammonium sulfate. After 16 h of cultivation, the cells were washed and transferred to a fresh mineral medium containing either glucose, lactose, ethanol or potassium acetate at 1% (w/v) as carbon sources or either ammonium sulfate, glutamate, glutamine or leucine at 40 mM, based on their molecular weight, as nitrogen sources. The cultures were incubated for further 16 h in rotating shaker at 30°C. These cultures were used to inoculate specific mineral media at 1/50 dilution for flask-batch experiments. In pH-controlled experiments, media cultures were buffered with 10 mM PIPES [Piperazine-N,N'-bis (2-ethanesulfonic acid)] at indicated pH values. Duplicate experiments were performed for each tested culture condition.

Enzyme analysis

Cell density was accompanied by optical density of the culture at 660 nm. Cell biomass was washed in buffer (100 mM tris pH 7.5 and 1.5% triton X-100) and suspended in 1/100 initial volume with the same buffer. The cells were broken with acid-washed glass beads with four cycles of 30 s vortexing and 30 s on ice. Supernatants were collected and centrifuged for 20 min at 12,000 g to remove cell debris. The cell-free extracts were used for enzyme activity as described by Norkrans and Tunblad-Johansson (10). One enzyme unit (U) is the amount of protein

capable of oxidizing 1 μmol NADPH/min at 30°C. For very low enzyme activity, milli-unit (mU; 10⁻³ U) was used. Protein concentration was measured by the Folin-Ciocalteu method (9). Enzyme specific activity referred to the amount of enzyme units per milligram of protein in the cell free extract. Residual glucose concentration in the supernatants was determined by the Dinitrosalicylic acid method (10), while residual ammonium concentration was measured according to Cataldo *et al.* (11). All reagents were purchased from Sigma-Aldrich Co.

Chemicals

Commercial YNB, yeast extract and peptone are from Difco Co. (Detroit, USA). Glucose and lactose are from Vetec (Rio de Janeiro, Brazil). Ethanol is from Merck S.A. (Rio de Janeiro, Brazil). Ammonium sulfate, amino acids, PIPES, potassium acetate and the chemicals for enzyme activity and protein, glucose and ammonia determinations are from Sigma-Aldrich Co. (St. Louis, USA).

RESULTS AND DISCUSSION

Cells of *K. marxianus* CBS6556 were batch cultivated in mineral medium containing glucose and ammonium sulfate as carbon and nitrogen sources, respectively, and the calculated maximum specific growth rate of 0.4 h⁻¹. During this period a very low NADP⁺-Gdh activity (<10 mU.mg⁻¹) was detected in cell-free extract (Fig. 1). However, when the residual ammonium

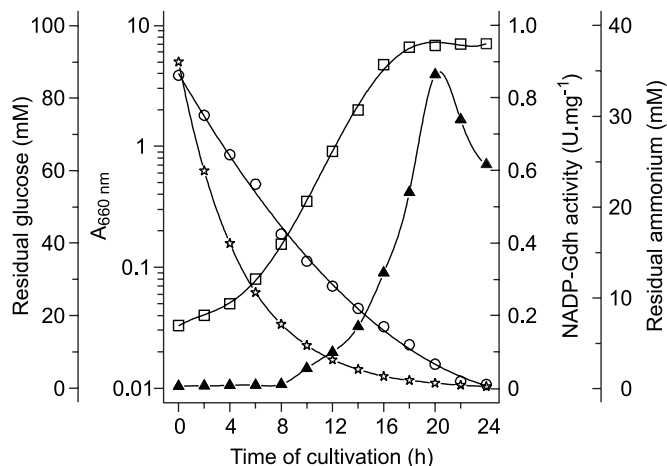


Figure 1. Flask-batch cultivation profile of *Kluyveromyces marxianus* CBS6556 grown in mineral medium containing 90 mM glucose (16 g.L⁻¹) and 38 mM ammonium sulfate (5 g.L⁻¹) as carbon and nitrogen sources, respectively. Symbols represent optical density at 660 nm (□), glucose consumption (○), ammonium consumption (☆) and NADP⁺-Gdh specific activity (▲). Each value represents the mean of two independent experiments.

concentration decreased below 8 mM it was possible to detect NADP⁺-Gdh activity in cell-free extracts, reaching a peak of 0.88 U.mg⁻¹ at 20 h of cultivation with low availability of ammonium (<0.6 mM) to sustain cell growth (Fig. 1). The decrease in the enzyme activity thereafter may be explained by the inability of NADP⁺-Gdh to trace the low level of intracellular ammonium. This result suggests that, similarly to *S. cerevisiae*, *K. marxianus* NADP⁺-Gdh activity primary functions to assimilate ammonium when its extracellular concentration is in a very narrow range.

The effect of initial ammonium concentration in the medium on the NADP⁺-Gdh activity was tested after growing yeast cells for 16 h as shown in Fig. 2. The highest enzyme activity was detected for cultures starting with 40 mM ammonium sulfate, which according to the fermentative profile in Fig. 1 should contain around 1 mM at 16 h of cultivation. The lower the concentration of ammonium sulfate at the beginning of cultivation, the lower enzyme activity was detected in cell-free extracts (Fig. 2). However, a short-term induction of the enzyme activity was observed 15 min after the addition of ammonium sulfate even at such low concentration of 0.5 mM in the medium (Fig. 2, insert). Additionally, cells cultivated at the highest initial

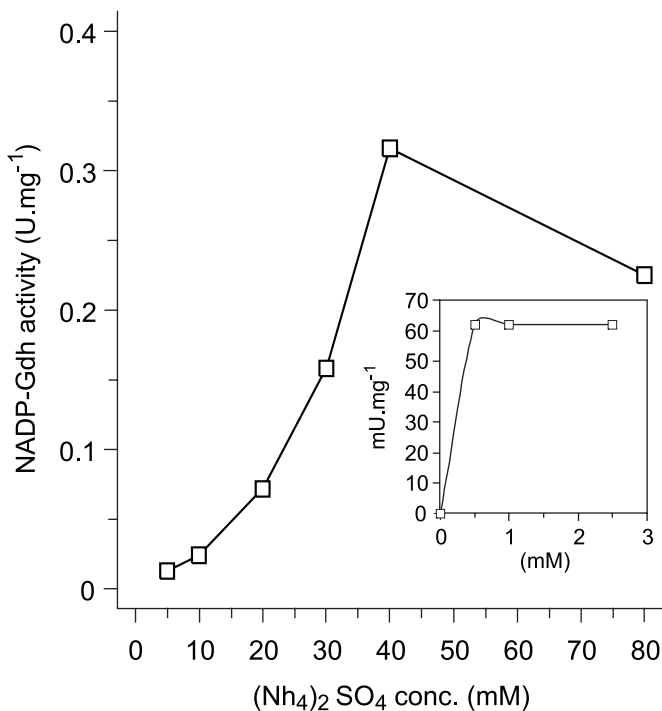


Figure 2. NADP⁺-Gdh specific activity of protein extracts from *K.marxianus* CBS6556 cells grown for 16 h in mineral medium containing 90 mM glucose and different ammonium sulfate concentrations. The insert represents the rapid induction of NADP⁺-Gdh activity 15 min after ammonium sulfate addition. Each value represents the mean of two independent experiments.

ammonium sulfate concentration (80 mM) showed the lowest enzyme activity (Fig. 2). A similar effect was observed for *S. cerevisiae* cells upon cultivation in external ammonium concentrations above 50 mM (3). In that case, the highest enzyme activity was detected at 24 mM ammonium sulfate, decreasing for higher ammonium sulfate concentrations. Bongonez *et al.* (3) showed that intracellular ammonium concentration increased in the presence of increasing extracellular ammonium concentrations, which could be involved in the decreasing of enzyme activity in *S. cerevisiae* cells. Moreover, steady-state *S. cerevisiae* cells showed higher NADP⁺-Gdh activity at higher dilution rates, as a consequence of the decrease in the residual ammonia concentration in the supernatant (13). This effect of initial ammonium concentration on the *K. marxianus* NADP⁺-Gdh activity was followed along the cellular growth. In cultures with 10 mM initial ammonium sulfate, the NADP⁺-Gdh activity exponentially increased up to 8 h of cultivation, followed with a decrease caused probably by nitrogen depletion in the medium (Fig. 3). Nonetheless, cultures with 40 mM initial ammonium sulfate showed biphasic behaviour with a linear increase for the first 4 h followed by an exponential increase.

An alternative way for testing the effect of ammonium concentration on the enzyme activity is to induce its intracellular

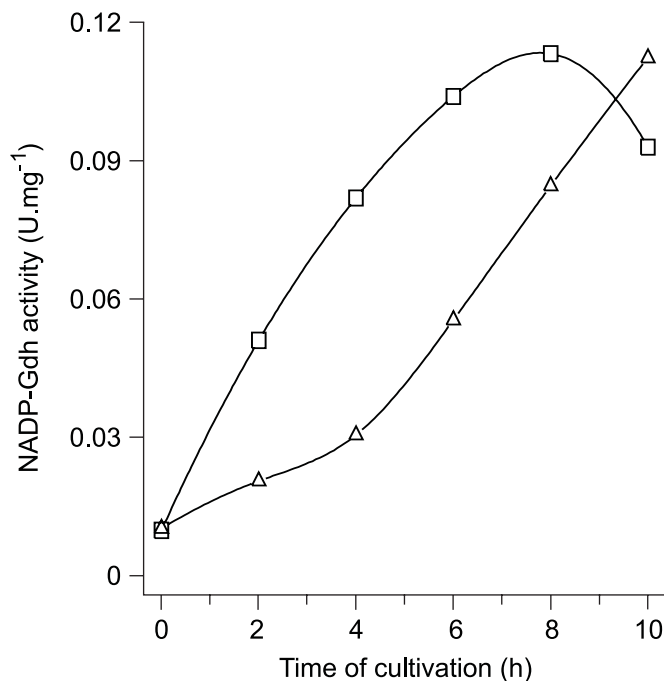


Figure 3. Kinetics of NADP⁺-Gdh activity along cultivation of *K.marxianus* CBS6556 cells in mineral medium containing 90 mM glucose in the presence of 10 mM (□) or 40 mM (△) ammonium sulfate. Each value represents the mean of two independent experiments.

accumulation. Ammonium uptake operates in yeast at very narrow pH range (5.5-7.5) (5). At low pH, ammonium ion (NH_4^+) formation is favoured and in yeast cells ammonium uptake is mediated by an H^+ -antiport transport systems at expenses of ATP (1 ATP per mol of NH_4^+). This explains the medium acidification observed when growing yeast cell on glucose and ammonium salt. However, at higher pH the ammonia (NH_3) formed accumulates in growing cells essentially by diffusion process (3). This methodology was used for further verification of the effect of high ammonium concentration on NADP⁺-Gdh activity. Cell cultivations were carried out in buffered mineral medium and the results are presented in Table 1. NADP⁺-Gdh activity decreased 6- and 10-fold when pH of medium was buffered at 5 or 7.5, respectively, comparing to a non-buffered condition. Bogonez *et al.* (3) observed a 10-fold increase in intracellular ammonia concentration of *S. cerevisiae* cells when medium pH was maintained at 5, which was followed by a 2-fold decrease in NADP⁺-Gdh activity. That decreasing in the enzyme activity was even higher by raising medium pH to 7. Our results indicate that *K. marxianus* NADP⁺-Gdh also negatively responds to the increasing in intracellular ammonium concentration.

The effects of different carbon sources on the NADP⁺-Gdh activity were also evaluated. *K. marxianus* cells showed significantly lower specific growth rate on ethanol when comparing to glucose (Table 2), which were practically the same

Table 1. Effect of medium pH on the NADP⁺-Gdh activity of *K.marxianus* CBS6556 cells.

Buffering ^a	Initial pH	pH of medium at harvest	NADP ⁺ -Gdh (U.mg ⁻¹)
Yes	7.5	7.0	0.03
Yes	5.0	5.0	0.05
No	6.3	3.8	0.31

^aCells were cultivated for 16 h in mineral media containing 90 mM glucose and 40 mM ammonium sulfate, buffered (yes) or not (no) at the beginning of cultivation with 10 mM Pipes-HCl. Each value represents the mean of two independent experiments.

values found for shake flask cultivation of *K. lactis* cells in both carbon sources (8). No effect on the cell growth was observed for lactose media. For cultivation on either glucose (respiro-fermentative carbon source) or ethanol (fully respiratory carbon source) no difference on the enzyme activity was observed (Table 2), suggesting that the enzyme is not subjected to glucose repression. On the other hand, the use of acetate as the single carbon source promoted a 3-fold decreased in the specific growth rate and a 10-fold decrease in the NADP⁺-Gdh activity (Table 2). These effects were also observed for *S. cerevisiae* cells (3). This raises two possible explanations: growth-association or substrate-repression. The dependence of the NADP⁺-Gdh activity on the growth rate could be discharged by the enzyme kinetics as shown in the Fig. 1 and the values attained on ethanol. A long-term exposure to high ammonium concentration, with consequent raising in the intracellular ammonium, seems therefore to be a better explanation. Contrary to found in the present paper (Fig. 1), NADP⁺-Gdh activity of *K. lactis* cells grown in mineral medium was higher at the beginning of cultivation and decreased along the cell growth (14).

Finally, it was investigated whether the same nitrogen-regulatory mechanism that takes place in *S. cerevisiae* can be applied for *K. marxianus*. A 3.5-fold reduction of the NADP⁺-Gdh activity was observed for cells growing on glutamate, followed by 2-fold decrease in leucine and 1.8-fold decrease in glutamine, when compared to ammonia-growing cells (Table 2). However, glutamine seems to repress *GDH1* gene expression more severely in *S. cerevisiae* cells than glutamate (7,13), while leucine promoted a 2-fold repression in the gene expression (7). The excess of leucine acts by repressing the enzymes of its first biosynthetic pathway, impairing α -isopropylmalate formation. This compound is essential for inducing *Leu3p* activator protein to binding *UAS_{LEU}* element in the promotor region of *GDH1* gene. Therefore, this gene is less induced in leucine-growing *S. cerevisiae* cells (7). Glutamine, and possible glutamate, exerts repression effect by activating the *Ure2p* repressor, which in turn inactivates *Gln3p* responsible for activating nitrogen-regulated genes in *S. cerevisiae* (13). This may be an indication that *K. marxianus GDH1* gene, which has not been cloned to

Table 2. Effect of different carbon or nitrogen sources on the specific growth rate and NADP⁺-Gdh specific activity of *K. marxianus* CBS6556 cells.

N-source (Mineral medium + 1% glucose)			C-source (Mineral medium + 40 mM $(\text{NH}_4)_2\text{SO}_4$)		
(40 mM)	(h ⁻¹)	Gdh (\pm SD)	(1%)	(h ⁻¹)	Gdh (\pm SD)
$(\text{NH}_4)_2\text{SO}_4$	0.40	0.36 (0.04)	Glucose	0.40	0.32 (0.03)
Glutamate	0.39	0.09 (0.03)	Lactose	0.42	0.31 (0.02)
Glutamine	0.40	0.22 (0.05)	Ethanol	0.27	0.33 (0.03)
Leucine	0.27	0.16 (0.01)	Acetate	0.13	0.02 (0.01)

date, may have UAS_{LEU} element in its promotor region, and may be regulated by the same regulatory pathway as *S. cerevisiae*.

The evidences presented here suggest that ammonium assimilation pathway of *K. marxianus* resembles more that of *S. cerevisiae* than its closely related specie *K. lactis*.

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RESUMO

Glutamato desidrogenase dependente de NADP⁺ da levedura *Kluyveromyces marxianus* responde à repressão catabólica de maneira similar à *Saccharomyces cerevisiae*

Glutamato desidrogenase dependente de NADP⁺ (NADP⁺-Gdh) constitui o primeiro passo enzimático no mecanismo de assimilação de nitrogênio em *Saccharomyces cerevisiae* e o conhecimento de sua regulação é chave na iniciativa de vários propósitos biotecnológicos, tais como a produção de proteína microbiana. A regulação da atividade NADP⁺-Gdh em células de *Kluyveromyces marxianus* foi avaliada a partir de diferentes condições de suprimento de amônia em cultivo em batelada. Os resultados mostraram que a atividade NADP⁺-Gdh de *K. marxianus* foi induzida em uma estreita faixa de concentração de amônia no meio, sendo reprimida tanto por altas concentrações deste composto quanto pelo produto glutamato. Esta atividade não está associada ao crescimento celular e deve funcionar principalmente no rastreamento de pequenas quantidades de amônia após a parada do crescimento celular. Isto demonstra que NADP⁺-Gdh não deve ser a principal enzima de assimilação de amônia em *K. marxianus*, como tem sido postulado para *K. lactis*, contudo deve estar submetida ao mesmo mecanismo regulatório encontrado em *S. cerevisiae*.

Palavras-chave: assimilação de amônia, atividade enzimática, glutamato desidrogenase, *Kluyveromyces marxianus*.

REFERENCES

1. Belem, M.A.F.; Lee, B.H. Production of bioingredients from *Kluyveromyces marxianus* grown on whey: An alternative. *Crit. Ver. Food Sci. Nutr.*, 38:565-598, 1998.
2. Bergemeyer, H.U. *Methods of enzymatic analysis*, 3rd ed., vol. VIII. Weinheim-Verlag Chemie, Berlin, 1985.
3. Bongonez, E.; Satrustegui, J.; Machado, A. Regulation by ammonium of glutamate dehydrogenase (NADP⁺) from *Saccharomyces cerevisiae*. *J. Gen. Microbiol.*, 131:1425-1432, 1985.
4. Cataldo, D.A.; Schrader, L.E.; Youngs, V.L. Analysis by digestion and colorimetric assay of total nitrogen in plant tissue high in nitrate. *Crop Sci.*, 14:854-856, 1974.
5. Dubois, E.; Grenson, H. Methylamine/ammonia uptake system in *Saccharomyces cerevisiae*: multiplicity and regulation. *Mol. Gen. Genet.*, 175:67-76, 1979.
6. Gonzalez-Siso, M.I.; Freire-Picos, M.A.; Ramil, E.; Gonsalez-Domingues, M.; Torres, A.R.; Cerdan, M.E. Respirofermentative metabolism in *Kluyveromyces lactis*: insights and perspectives. *Enz. Microbial Technol.*, 26:699-705, 2000.
7. Hu, Y.; Cooper, T.G.; Kohlhaw, G.B. The *Saccharomyces cerevisiae* Leu3 protein activates expression of *GDH1*, a key gene in nitrogen assimilation. *Mol. Cell. Biol.*, 15:52-57, 1995.
8. Kiers, J.; Zeeman, A.M.; Lutik, M.A.H.; Thiele, C.; Castrillo, J.I.; Steensma, H.Y.; van Dijken, J.P.; Pronk, J.T. Regulation of alcoholic fermentation in batch and chemostat cultures of *Kluyveromyces lactis* CBS 2359. *Yeast*, 14:459-469, 1998.
9. Magazanik, B. Regulation of nitrogen utilization. In: Jones; Pringle; Broach (eds). *The molecular and cell biology of the yeast Saccharomyces cerevisiae: gene expression*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1992, p.283-317.
10. Norkranz, B.; Tublad-Johanson, I. Changes in free amino acids content and activities of amination and transamination enzymes in yeast growth on different inorganic nitrogen sources, including hydroxylamine. *Antonie van Leeuwenhoek*, 47:217-230, 1981.
11. Oliver H.; Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193:265-275, 1951.
12. Romero, M.; Guzmanleon, S.; Aranda, C.; Gonzalez-Halpen, D.; Valenzuela, L.; Gonzalez, A. Pathways for glutamate biosyntheses in the yeast *Kluyveromyces lactis*. *Microbiology*, 146:239-245, 2000.
13. Ter Schure, E.G.; Silljé, H.H.W.; Raeven, L.J.R.M.; Boonstra, J.; Verrips, C.T. Nitrogen-regulated transcription and enzyme activities in continuous culture of *Saccharomyces cerevisiae*. *Microbiology*, 141:1101-1108, 1995.
14. Valenzuela, L.; Guzmanleon, S.; Coria, R.; Ramirez, J.; Aranda, C.; Gonzalez, A.A. NADP-glutamate dehydrogenase mutant of the pet-negative yeast *Kluyveromyces lactis* uses the glutamine synthase glutamate synthetase pathway for glutamate biosynthesis. *Microbiology*, 141:2443-2447, 1995.
15. Zeeman, A.M.; Kuyper, M.; Pronk, J.T.; van Dijken, J.P.; Steensma, H.Y. Regulation of pyruvate metabolism in chemostat cultures of *Kluyveromyces lactis* CBS 2359. *Yeast*, 16:611-620, 2000.