Growth of *Pichia guilliermondii* strain Levica 27 before different energy sources and nitrogen

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The growth of *Pichia guilliermondii* yeast Levica 27 strain was studied for its use as microbial additive in ruminant animals. Therefore, yeast growth in NRF solutions and malt extract were compared. Then, their development was evaluated when carbohydrates and nitrogen sources varied in the formulation of NRF solution. In sugars, the glucose was substituted by sucrose and lactose and in case of nitrogen the tryptone was substituted by ammonium sulfate, casein and urea. There was not interaction between treatments and the time of study for the count of Levica 27cells in malt extracts and NRF solutions, and there were not differences between both means. In NRF mean, the best growths were achieved when glucose and sucrose were used at 72h and the last one decreased the pH of the mean ($P < 0.001$).The casein and urea caused higher growth ($P < 0.001$) after 48h, but urea caused increase of pH. It is concluded that the strain Levica 27 growth was higher before glucose or sucrose, casein or urea. Studies are recommended to design an economical mean of culture with national sources that allows the obtaining of a preparation with Levica 27 with additive effect on ruminants.

Key words: *Pichia guilliermondii*, *carbohydrates, nitrogen*

The addition of yeasts to the diet of ruminant animals exerts favorable effects on the microbial population and on the fermentative indicators of the rumen and as consequence; it improves health and animals productivity (Bruno *et al.* 2009 and Ibrahim *et al.* 2012).

The Instituto de Ciencia Animal (ICA) from República de Cuba has a collection of yeasts isolated from the ruminal ecosystem that do not belong to Saccharomyces genus. *In vitro* studies demonstrated that the strain from the mentioned collection called Levica 27, which belongs to *Pichia guilliermondii* specie, showed higher potentiality for its use as additive in ruminants that consume fibrous diets, when was compared with a *Saccharomyces cerevisiae* strain(Marrero *et al.* 2014).

These results justify the search of an appropriate culture mean, economically feasible, for the obtaining of an additive product to pilot scale, that allow to show the activator effect of the strain on animals in production.

It is known that the design of the culture mean is one of the most important tasks inside the biological technology. According to Winkler (1998), in the total cost of the biotechnical products, raw matters can represent between 30 and 80 %.The composition of the culture mean it also has to satisfy all the nutritional requirements from the microorganism. In this sense, yeasts are able to survive under adverse conditions, behaving as facultative anaerobes. They are also easy to cultivate whether in the laboratory as to industrial scale, using a culture mean that includes sugars, minerals salts, and a small quantity of nitrogen source (Alvarez 1995).

The objective of this study was to evaluate the growth of *Pichia guilliermondii* strain Levica 27 before different energy sources and nitrogen, for a later design of a culture mean.

Materials and Methods

Biological material and culture means. The strain Levica 27, belonging to *Pichia guilliermondii* specie, located in Gen Bank, with number JF894143 was used. This strain belongs to yeasts collection from ICA (RYCASI), with registration number 980 in World Data Center for Microorganisms (WDCM).

For the research the culture means extract malt and NRF solution were studied , of Caldwell and Bryant (1996), modified by Elias (1971), since although the first one is specifically used for fungi and yeasts culture, it does not have the mineral solutions of the second in its formulation, which could favor yeast development.

The NRF mean is used for ruminal yeasts culture therefore was eliminated from its composition the cellobiose, soluble starch, sodium lactate, hemina, volatile fatty acids, HCL-cysteine, $Na_2S.9H_2O$ and $Na_2 HCO_3$.The composition of the used mean is showed in table 1.

The mineral solution A contains, per 1,000 mL, K_2HPO_4, 3.0 g. The mineral solution B contains, per 1,000 mL: KH_2PO_4, 3.0 g; $SO_4 (NH_4)2$, 6.0 g; NaCl, 6.0 g; $MgSO_4 \bullet 7H_2O$, 0.6 g and CaCl2, 0.6 g.

*Preparation of yeast inoculu*m. A yeast culture obtained at 24h was used, from which a lop was taken, and was diluted in 10mL of malt solution extract. It was incubated at 30 ºC during 24 h for their reactivation. Then, from this culture, 0.5mL was inoculated in an erlenmeyer of 100mL with 50 mL of the same mean and was incubated under the same conditions. This constituted the inoculums for the studies and shows a concentration of 57.1 x 10^{-7} cel•mL^{-1} of live cells.

Experimental procedure. The growth of the yeast

Table 1. Composition of NRF mean, modified to determine the requirements of Levica 27 strain

Ingredients	Quantity for final volume of 100 mL
Mineral solution A	15.0 mL
Mineral solution B	15.0 mL
Glucose	0.4 g
Tryptone	0.2 g
Yeast extract	0.25 g
Cu SO_4 • 5 H_2O [0.15%, LW^{-1}, in solution]	1.0 mL
Mn Cl_2 •4 H_2O [0.1 %, LW^{-1}, in solution]	1.0 mL
Co Cl_2 [0.1 %, LW^{-1}, in solution]	0.1 mL
Fe SO_4•7H_2O [0.34% LW^{-1} in solution]	1.0 mL
Distilled water	67.0 mL

strain in the extract malt solution and the modified NRF solution was first compared. The first mean was used, specifically, for fungi and yeasts culture, but it not have in its formulation the minerals solutions of the second one. For this, 0.1mL of the inoculum above cited in test tubes that contained 10mL of both solutions was added. Four tubes per treatment for every study hour were used. Tubes constituted the experimental unit. At 0, 4, 8,12,24,36 and 48 h the pH and optical density (O.D) were determined. Cell counts were also made in Neubauer camera. For this, yeasts were tinted according to Painting and Kirsop (1990) method.

Later on the strain growth was studied in NRF solution when changing sugars and nitrogen sources, according to the study. In the first case, glucose was substituted by sucrose and lactose and for nitrogen sources, the ammonium sulfate found in the B mineral solution was removing to included tryptone (144mg), casein (144mg) and urea (24.4).For this, same as in the curves study, 0.1mLof the inoculums was added in test tubes that contained 10 mLof both solutions. Four tubes per treatment for every study hour were used. Tubes constituted the experimental unit. At 0.48 and 72 h the pH and O.D were determined.

The pH was measured with a portable potentiometer HANNATM., model HI 9017, ± 0.1 precise units. The O.D was measured by colorimetry, with an Espectrophonometer HACH, model DR 5000, at wave longitude of 530 nm.

Statistical analysis. A completely randomized design was used, with four repetitions per treatment in every study hour. The experimental results were analyzed with the InfoStat statistical package, version 1.0 (Balzarini *et al.* 2001).In the microorganism counts, data were transformed according to LN, to assure variance normality. The comparison Duncan test (1955) was applied, in necessary cases to distinguish differences between means.

Results and Discussion

To growth analysis, yeasts have high similarities with heterotrophic bacteria than with the filamentous ascomycetes and basidiomycetes with which are taxonomically related. Therefore, if are excluded some yeasts genus similar to filamentous fungi, can be said that its growing has an excellent approximation to a homogeneous population of unicellular heterotrophic organisms (Casadesus *et al.* 1985).

When analyzing the curves that create the cell count of Levica 27 in solutions of extract malt and NRF (figure1) was proven the similarity between them. The statistical analyses showed that there was not interaction between treatments and the study time. There were not differences in the number of cell that grew in both means (table2), showing that the NRF mean is appropriated for the culture strain in study and the evaluation of its growth before different carbohydrates and nitrogen sources. However, in table 3 are show the significance differences ($P < 0.001$) that were found in the time, logically by the increase of cell numbers that shows the microorganisms, due to its reproduction, when they are in a culture mean with the necessary nutrients for this process.

The previous results, in both culture means, verified the existence of two from the four phases described by Mateos (2005) for the microorganism growths in general: lag phase and exponential phase or logarithmic.

The existence of the stationary and decline or death phases was not verified, since there was increase of cell counts up to the 48h. Apparently, the nutrients quantity in the mean was enough to achieve a yeast growth up to this hour.

The growth Lag phase of Levica 27 was extended up to the 8h.In the adaptation and recovery periods in which this phase is divided, first the cells are adapted to the new mean conditions, and could happen decrease in viable cells percentage. Later on begins to increase the cellular division speed.

The exponential growth phase is show from 8h up to the 48 h. To this regard, Mateos(2005) show that, in this period, nutrients are used quickly and the number of cells is increased. As consequence, final metabolites that can bring changes in the pH are accumulated. This statement is corroborated in table 4, which shows the pH and OD of Levica 27 culture in the study means. In both indicators, there were interaction between time and treatments. Decrease of the Ph was observed, while OD increases.

It should highlight that yeast grew high to pH in the NRF solution (from 6.0 to 4.41) regarding to malt solution (from 4.22 to 4.54).To this regard, Carrillo (2003) explained that yeasts accept a pH range between 3 and 10, but prefers a lightly acid habitat, with pH from 4.5 to 6.5, corresponding with the obtained values in both cultures.

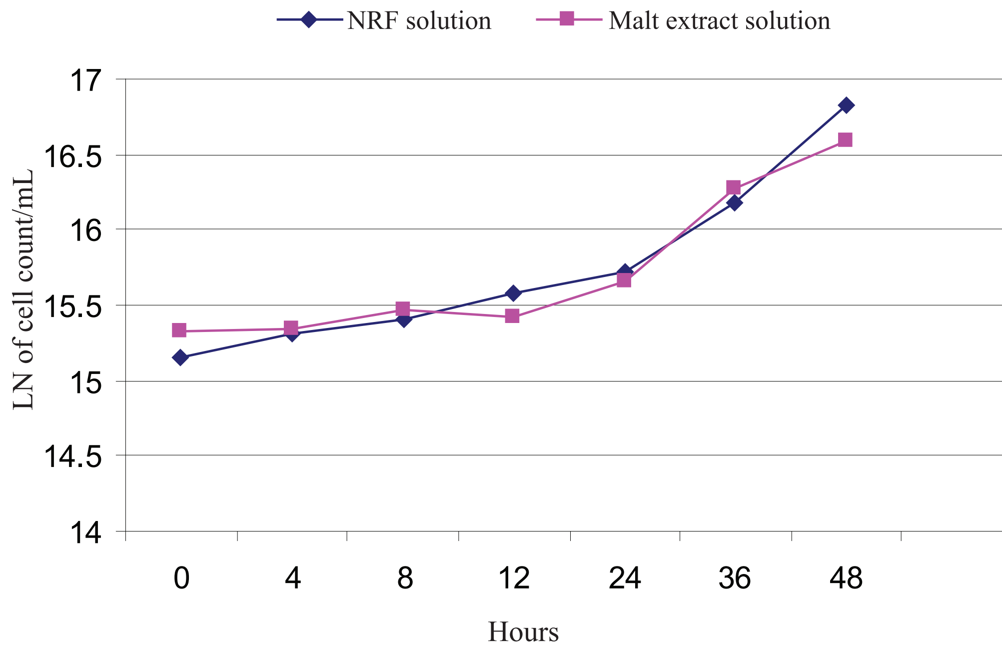

Figure 1. Growth curve of Levica 27 strain in modified NRF and malt extract solution

Table 2. Effect of the treatment on cell counts of Levica 27 (x 10^{-7} cell•mL^{-1})

	Treatments		SE
	Malt extract solution	NRF solution	0.04
Yeast cells	15.71	15.72	

Tabla 3. Effect of time on cell counts of Levica 27 (x 10^{-7} cell•mL^{-1})

	Time (h)							SE
	0	4	8	12	24	36	48	0.08***
Yeast cells	15.23^{a}	15.32^{a}	15.41^{ab}	15.47^{ab}	15.64^{b}	16.22^{c}	16.71^{d}	

^{abcd}Different letters show significant differences ($P \leq 0.05$)
***$P < 0.001$

Table 4. Performance of pH and O.D of Levica 27 strain on malt extract and NRF solutions

Time (h)	pH		O.D	
	NRF solution	Malt solution	NRF solution	Malt solution
0	6.41^{g}	4.54^{c}	0.015^{a}	0.013^{a}
4	6.40^{g}	4.52^{c}	0.015^{a}	0.010^{a}
8	6.23^{f}	4.49^{c}	0.017^{ab}	0.023^{ab}
12	6.18^{f}	4.48^{c}	0.025^{ab}	0.037^{b}
24	6.00d	4.38^{b}	0.057^{c}	0.073^{c}
36	6.10^{e}	4.32^{b}	0.104^{d}	0.126^{e}
48	6.08^{e}	4.22^{a}	0.102^{d}	0.182^{f}
SE	0.0061***		0.0017***	

^{abcdefg}Different letters show significant differences ($P \leq 0.05$)
***$P < 0.001$

The microorganism growth can be verified by direct cell count method, and by indirect methods, as the cloudiness measurement through O.D (Pandey *et al.* 2001).For this reason, studies of O.D lineal regression adjusted to cell count for means in study were carried out and the corresponding equations were stated:

Extract malt solution

Cell count =3.21 x 106 + 67.32 x 106 (O.D)

NRF solution

Cell count= 2.84 x 106 + 110.07 x 106 (O.D)

The results showed high regression coefficients of R^2= 0.90 in the extract malt solution and R^2= 0.60 in NRF solution, with $P < 0.001$in both cases.

This showed the possibility to use the O.D as indicator of Levica 27 growth in these culture solutions.

Microorganism growth dynamics depends on multiple factors. Besides the genus, specie and, specifically, the strain, variability is observed when different conditions, culture means and inoculum doses are used. This statement is confirmed by Tai Shin *et al.* (2002), Castillo (2009) and Angulo (2012), who showed heterogeneous results in the growth dynamics of Candida norvegensis, due to the use of culture mean and different inoculums doses. Everything seems to indicate that doses of inoculum inclusion were decisive in the differences among the three studies. The first two studies included 20 and 5% respectively, and the third one, 0.1%.

Although this study was carried out with a strain of the P.guilliermondii specie, the inoculum dosis was the same used by Angulo (2012). Probably the amount of inoculum was very low, which caused that Lag growth phase were extended up to the 8 h.

Results showed the possibility to study the growth of *Pichia guilliermondii* strain Levica 27, in NRF solution with different energy sources and nitrogen. Figure 2 shows the OD values of the strain culture in NRF solution for the three carbohydrates used as energy sources. All sugars were used by the yeast, but the best growths were reached up to the 48 h, when glucose and sucrose were used, although with sucrose at 48h the same O.D values were reached as with glucose at 72h. This should be taken into account due to the lower time used by the strain for growing with this carbohydrate.

Similar studies, in those the NRF mean was used to determine the strain requirements from the same genus, showed that this one mostly used glucose as energy source, when was compared with sucrose and lactose (Angulo 2012).This shows the specificity that exits in yeast species, as for the use of carbohydrates sources.

The previous has been confirmed by Kurtzman and Fell (1998), whose describe the yeast capacity to use a wide range of compounds, among them carbohydrates that lend them a significant value to be use in the industry and agriculture.

In the means that contained glucose and sucrose had pH decrease (table5) at 48h, which extended with sucrose mean at 72h.However, this one stayed in the optimal range described for yeast growth(Carrillo, 2003) and the described result is confirmed in table 4.

Sánchez *et al.* (2007), when studying the pH influence in the growth of a P. guilliermondii strain, determined that there were not differences in this yeast growing in the pH interval between 5 and 7, in which the higher biomass production were reached. They also verified that they can also work with pH from 4 to 4.5, without a marked affectation in growth. However, at PH 3, there was a radical affectation of growth, due to the high acidity of the mean.

Contrary to previous researches, with yeasts from the same genus, but from the I. orientalis specie, 92% of

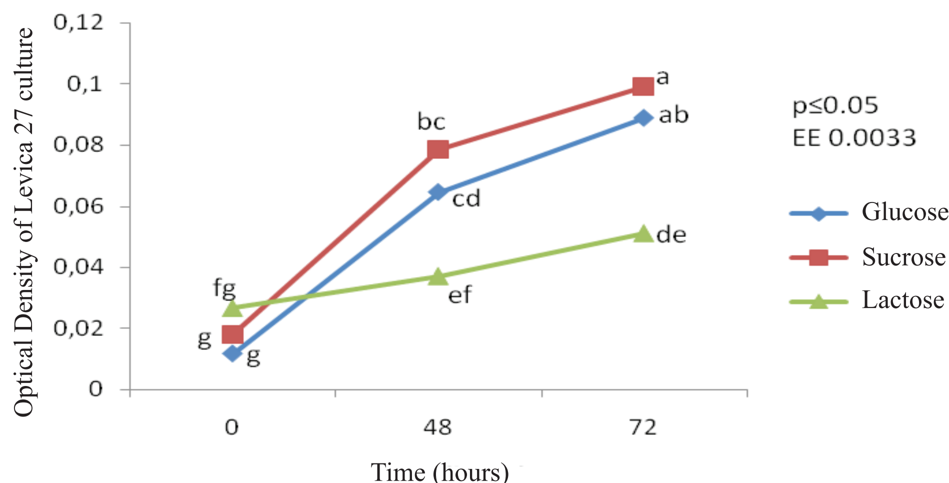

Figure 2. Effect of different carbohydrates on growth of Levica 27 culture

Table 5. Effect of different carbohydrates on pH of Levica 27 culture

	Carbohydratess	Time (h) 0	48	72	±SE
pH	Glucose	6.40^{b}	6.12^{d}	6.12^{d}	
	Sucrose	6.42^{ab}	5.91^{e}	5.72^{f}	0.118*
	Lactose	6.43^{ab}	6.47^{a}	6.25^{c}	

[abcdef]Different letters show significant differences ($P \leq 0.05$). ***$P < 0.001$

survival to PH 3 was maintained (Tai Shin *et al.* 2002). From there, the importance of the specific studies in each strain, when is intended to use yeasts in ruminant animals feeding. The hardies to low pH could be more effective in diets with concentrates, in those the presence of carbohydrates of easy fermentation decrease the rumen pH.

Calderón *et al.* (2005) suggested that yeasts can get nitrogen to synthesize unicellular protein and the common sources are ammonium, urea and amino acids (Déak and Beuchat, 1996).This was evidenced in this study, with Levica 27 growing, presenting different nitrogen sources (figure 3).It can verify that O.D values, when using casein, urea and triptane, they duplicate those that are obtained in ammonium sulfate presence. Regarding, Villamil and Zapata (1999) mention that all yeasts can use nitrogen in ion ammonium way, either in chlorides, nitrates, sulfates, ammonium phosphates, and can use the sulfur from the ammonium sulfate for the synthesis of some amino acids(Sarmiento and Herrera 2003).

If the results of figure 2 and 3 are analyzed it could recommend different combinations of carbohydrates and nitrogen sources taking into account their market prices.

The presence of all nitrogen sources caused gradual decrease of the pH, at 48 and 72 h (table 6), and the urea maintained values over the optimal for yeasts. Although the growth was not affected, this aspect should be kept in mind if it chosen the urea as source in culture mean to the studied strain. Nevertheless, Sánchez *et al.* (2007) designed a mean of simple culture for P. guilliermondii, without yeast extract, that contained molasses as carbon source and urea as nitrogen source. These authors obtained high biomass levels in submerged cultures as in fermentation in solid state.

It is concluded that the growth of *Pichia guilliermondii* strain Levica 27 was higher in presence of glucose or sucrose and casein or urea, when was compared with other energy and nitrogen sources, respectively. It is recommended to carry out studies to design an economical culture mean with energy and nitrogen national sources that allow the obtaining of a preparation with Levica 27 to make in vivo studies and check the additive strain effect.

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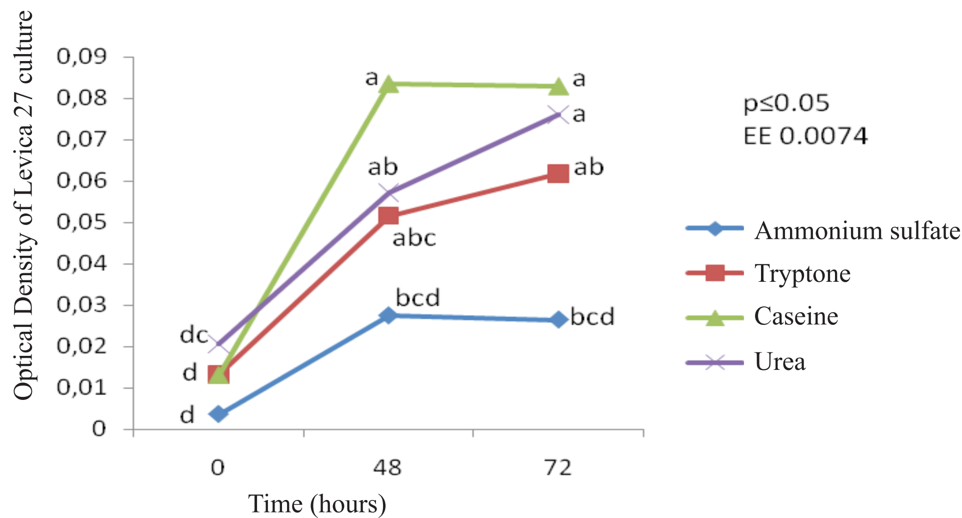

Figure 3. Effect of different nitrogen sources on Optical Density of Levica 27 culture

Table 6. Effect of different nitrogen sources on the pH of Levica 27 culture

Fuente de nitrógeno	Tiempo (h)			±EE
	0	48	72	
Sulfato de amonio	8.61[ab]	8.51[bc]	8.50[c]	0.0218*
Triptona	8.65[a]	7.90[e]	7.68[f]	
Caseína	8.46[c]	7.64[f]	7.60[f]	
Urea	8.52[bc]	8.20[d]	8.10[d]	

[abcdef]Different letters show significant differences ($P \leq 0.05$)

*0.05

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