Growth dynamics and metabolites produced in the fermentation of *Candida norvegensis* yeast

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An experiment was conducted to know the growth dynamics and metabolites produced by a yeast strain with potentialities as activator of the ruminal fermentation. The strain belongs to *Candida norvegensis* and is called Levazoot 15. A completely randomized design, with four repetitions in each study hour, was applied. The variables optic density, biomass, brix degrees, lactic acid and ammoniac nitrogen were determined. The results show that the performance of the optic density and that of the biomass were similar in time. The higher values were reached at 36 h (1.75 and 0.024, respectively). Consequently, the brix degrees diminished from 1.65 at 0 h, up to 0.73 in the 36 h. The levels of ammoniac nitrogen were reduced from 0.70 mM to 0.32 mM in the 12 h. The pH diminished up to the 12 h, but increased slightly from 16 h on. Similar performance was observed in the concentration of lactic acid, diminishing from 3.26 µg to 1.196 µg, for increasing later from 3.244 µg to 36 h. It is concluded that the Levazoot 15 (*Candida norvegensis*) strain showed its maximum growth at 16 h, with pH of 4.17 under the conditions of this study. This is the proper time to use the yeast culture and prove its effect as ruminal activator.

Key words: yeast, dynamic, growth, *Candida norvegensis*.

The feeds production that favors animal production and do not compete with human feeding is one of the main challenges in present cattle rearing. Under these conditions, alternatives to favor the animals' productivity are searched. In the higher part of Mexico, animal feeding is based on pastures and forages of low nutritive value, which do not cover the requirements for maintaining the animals. On this context, the studies are addressed to the use of antibiotics, supplements and additives to improve the ruminal environment and increase the feeding efficiency.

In the case of microbial additives, the yeast *Saccharomyces cerevisiae* and the conidial fungus *Aspergillus orizae*, are within the most beneficial microorganisms for these purposes. They are capable of increasing intake (Erasmus *et al*.1992), the number of cellulolytic bacteria in the rumen (Dawson 1987), the total bacteria and the concentration of short-chain fatty acids (Beharka and Nagaraja 1991). The ruminal pH also increases and the concentration of lactic acid diminishes (Williams *et al*. 1991). On this respect, Wiedmeier *et al*. (1987), Williams *et al*. (1991) and Wohlt *et al*. (1998) demonstrated that the inclusion of preparations with these microorganims increases the ruminal degradability of the ADF and nitrogen (Doreau and Jouany 1998). Besides, milk production increases (Carro *et al*. 1992, Sievert and Shave 1993 and Kung *et al*. 1997) and its chemical composition improves (Yu *et al*. 1997).

It has been proved that strains different from *S. cerevisiae* have potentialities for their use as ruminal activators of high-fiber diets (Lee *et al*. 2000). Castillo (2009) demonstrated in *in vitro* studies that *Candida norvegensis* stimulated the populations of cellulolytic fungi and increased the concentrations of short-chain fatty acids in the DM digestibility of oat straw. For this reason, further studies to increase the knowledge about this yeast are necessary.

The objective of this study was to know the growth dynamics and metabolites produced by the *Candida norvegensis* strain during its fermentation.

Materials and Methods

Localization of the study area. The experiment was conducted in the animal nutrition laboratory of the Husbandry and Ecology Faculty of the Autonomous University of Chihuahua (AUCH), located in the km 1 of the peripheral Francisco R. Almada.

Biological material and culture medium. The strain yeast Levazoot 15 (*Candida norvegensis*) from the collection of the UACH was used, with record number in the Gen Bank: JQ519367.1 GI: 386785959. It was isolated and indentified in previous studies (Castillo 2009) and was kept viable throughout periodic re-culturing in malt extract solution (MES). It was preserved in refrigeration at temperature of 4 °C.

Experimental procedure. For studying the growth dynamics of *Candida norvegensis* yeast, flasks of 250 mL with gauze cap containing 50 mL of the malt extract solution medium were used. They were inoculated with 0.1 % of *C. norvegensis*. The flasks were incubated at 30˚C in shaker model I2400, with

agitation of 120 rpm. In each hour of study (0, 4, 8, 12, 16, 20, and 36 h), four flasks were randomly moved away. From them, samples were taken to determine the optic density, biomass, brix degrees, pH, lactic acid and ammoniac nitrogen.

Obtaiment of the inoculum. A sample of the fresh culture of the strain in wedge shape was taken and dissolved in a test tube with 10 mL of MES, incubated for 24 h at 30˚C to obtain a pre-inoculum. Later, 0.5 mL of the pre-inoculum were taken and added to a flask with 50 mL of the MES, incubated at 30˚C with agitation of 120 rpm to obtain enough culture of the *C. norvegensis* strain. This was the inoculum used in the study.

Determinations in the study. The pH was measured with a portable potentiometer HANNATM, model HI 9017, ± 0.1 units precise. The methodology described by Rodríguez *et al*. (2001) was considered. The brix degrees were determined with a refractometer HANNA, model HI 96801. The optical density (OD) was measured by colorimetry with a spectrophotometer HACH, model DR 5000, at wave longitude of 530 nm. In order to determine the $N\text{-}NH_3$, colorimetry method described by Broderik and Kang (1980) was used.

For determining biomass, 10 mL of each sample were previously collected in plastic test tubes. They were placed in an oven at 60 °C. The sample was centrifuged in the tube twice; at 3000 rpm for 30 min. The granulate was suspended again with 10 mL of distilled water. The remaining liquid was discarded and the granulate was dried in an oven at temperature between 50 and 60 °C. It was removed at 24 h and weighed. The biomass was determined by weight difference.

Experimental design and statistical analysis. A completely randomized design with seven treatments was used. Time was considered as treatment, with four repetitions per hour assigned to be measured. All the flasks (28) used in the experiment were considered as experimental unit. The effect on the response variables was measured through the following model:

$Y_{ij} = \mu + t_i + \varepsilon_{ij}$

where:

Y_{ij} is variable of response

μ is general mean

t_i is effect of the i-th treatment (time)

ε_{ij} es error

The statistical analysis was conducted through the GLM procedure of the (SAS 2004). The method LSMEANS was applied for the comparison between means of the different variables to be measured in time.

Results and Discussion

The growth of a microorganism can be confirmed either by direct methods like cell counting or determination of the dry weight or by indirect methods like the turbidness measuring through the optic density (Pandey *et al*. 2001). Both methods were conducted in this study to assess the growth of Levazoot 15 yeast.

The results are shown in figures 1and 2, where the increase up to the 36 h is evidenced, which was more accelerated from the 0 h to the 12 ($P<0.05$), expressing the exponential or logarithmic growth phase. From 12 h on, there was a slower increase of both measuring, showing the presence of the growth stable phase, according to Mateos (2005). However, for demonstrating the lag or adaptation phase, it is more evident on the performance of the OD, from the 0 to the 4 h. This differs from the biomass curve, where such phase was not corroborated. The declination phase was not evidenced either in none of the two curves.

Studies conducted by Tai Shin *et al*. (2002) with a strain of *Issatchenkia orientalis*, cultured in discontinuous fermentation in glucose medium with inoculum at 20 %, maximum biomass values were obtained at 8h, when they coincide with minimum glucose values. The results were sustained by the counting of total and viable cells observed in this time and from that time on up to 12 h. The growth stable phase was observed.

If comparing the results of the previous study with those found in the present one, several causes that could explain the lack of declination phase could be identified. Probably, the amount of inoculum used (0.1%) was very low, extending the growth phases up to the 36 h and reaching the maximum biomass values between the 16 and 20 h.

Although this study did not include viable cell counting, the OD and biomass curves show the exhaustion of the medium nutrients from 12 h on. The presence of waste products, released during the exponential growth phase, influenced on the yeast growth.

Table 1 shows the results of the brix degrees, pH, $N\text{-}NH_3$ and lactic acid, corresponding to the yeast culture in time. The accelerated growth of the OD and biomass justify that the brix degrees diminished in time with significant differences ($P < 0.05$). This indicates that yeasts consumed the sugars of the medium very fasts up to the 12 h. these results are similar to those obtained by Tai Shin *et al*. (2002).

According to Déak and Beuchat (1996), yeasts ferment few sugars, mainly hexoses and oligosaccharides, but the compounds range they are able to assimilate is much wider. Pentoses, alcohols, organic acids, amino acids and glycosides are also included. This also explains the diminishing of brix degrees by the use of sugars for obtaining energy and the complete formation of cell protoplasm (Slatter and Esdale 1968).

The pH values diminished up to the 12 h, from when they increased up to the 36. This perfomance was due, probably, to the production of organic acids by the yeast (Nisbet and Martin 1991 and Flanzy 2003). They are segregated to the extracellelular medium up to the beginning of the stable phase. Everything seems to indicate that, from 16 h on, the microbial decomposition

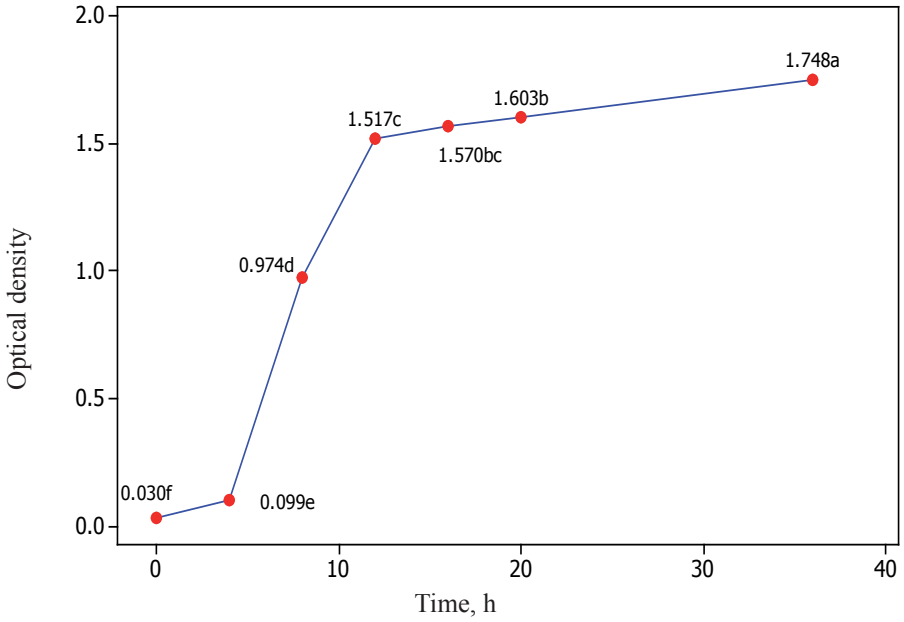

Figure 1. Performance of the optical density on the growth dynamics of *C. norvegensis* yeast

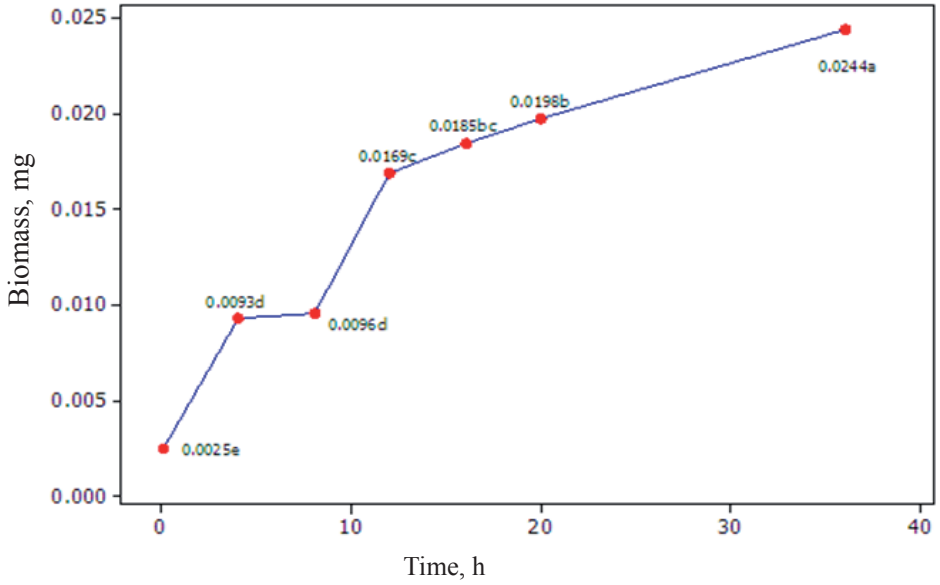

Figura 2. Performance of the biomass on the growth dynamics of *C. norvegensis* yeast

Table 1. Square minimum means (±SE) of the brix degrees, N-NH_3 and pH in different times of the dynamics

Variables	Time in hours							SE ±
	0	4	8	12	16	20	36	
Brix degrees	1.65^{a}	1.80^{a}	1.30^{b}	0.93^{c}	0.90^{cd}	0.75^{cd}	0.73^{d}	0.043
pH	4.67^{a}	4.59^{a}	3.95^{d}	3.97^{d}	4.17^{c}	4.23^{c}	4.39^{b}	0.023
N-NH_3 (mM)	0.70^{a}	0.54^{b}	0.43^{c}	0.32^{d}	0.30^{d}	0.31^{d}	0.31^{d}	0.012

^{abcd}values with different letters per row differ at $P < 0.05$

or the use of anionic compounds of the medium made it more alkaline (Stanier *et al*. 1992).

Similar performance was shown by the N-NH_3 levels that reduced up to the 12 h and kept low up to 36 h ($P > 0.05$). The cause of this N-NH_3 diminishing is assumed to be because the yeasts used the nitrogen of the medium for the cell protein synthesis (Calderón *et al*. 2005), corresponding with the obtained growth curves.

The results of this experiment make possible to continue searching on the introduction of an additive product from the Levazoot 15 strain, for its application on ruminants' feeding. Designing an economically

feasible new culture medium making possible obtaining this product at pilot scale for its introduction in the diet of different species is necessary.

It is concluded that the strain Levazoot 15 (*Candida norvegensis*) showed, under the conditions of this study, its maximum growth at 16 h, with pH of 4.17. This is the proper time for using the yeast culture and proves its effect as ruminal activator.

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