Growth of *Candida norvegensis* (strain Levazoot 15) with different energy, nitrogen, vitamin, and micromineral sources

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Abstract

To examine the growth of *Candida norvegensis* (strain Levazoot 15), four experiments were conducted with different sources of energy, nitrogen, vitamins, and microminerals. Optical density was used as an indirect measure of strain growth in a fully randomized factorial design, in which principal factor A was the source of energy, nitrogen, vitamins, or microminerals and principal factor B was the measurement time point (0, 20, or 40 h). The results showed that the yeast strain used glucose (primarily sucrose and lactose) as the energy source and tryptone as the nitrogen source. The addition of B-complex vitamins or microminerals was not necessary for strain growth. It is concluded that the strain Levazoot 15 preferentially utilizes glucose as a source of energy, tryptone as a source of nitrogen and manganese as a mineral source, and that no vitamin source was necessary for growth.

Keywords Yeast · Growth · Requirement · Nutrient

Introduction

A key challenge for nutritionists is the search for productivity improvement alternatives in livestock production. Studies of ruminant digestive processes have focused on microbial additives to increase digestive efficiency [1, 2]. Numerous studies have shown that the addition of yeasts, such as *Saccharomyces cerevisiae*, to ruminant diets increases digestive efficiency [3]. Strains such as *Issatchenkia orientalis* DY252 have also shown potential for use as additives [4], while a strain such as *I. orientalis*, *Rhodotorula mucilaginosa*, and *Pichia guilliermondii* strains greatly stimulated in vitro gas production from fibrous substrates [5]. Similarly, the Levazoot 15 strain of *Candida norvegensis* has shown

promise as a rumen fermentation activator in vitro and in situ, growth of ruminal microbial populations, increasing concentrations of short-chain fatty acids and in vitro digestibility of dry matter in high-fiber diets, and reducing methane production [6–8]. In a recent study, Marrero et al. [9] corroborated the strain's positive effects, demonstrating that it increases in vitro total gas production for various feeds. These findings suggest a need to study the growth requirements of this yeast strain, for development of an economical culture medium that will lead to large-scale biomass production, and to evaluate the strain's effect on livestock productivity. Yeasts, like all living organisms, require certain nutrients, such as carbon [10], hydrogens, oxygen, nitrogen, and phosphorus. They also require optimal environmental conditions for growth and reproduction [11]. The objective of this study was to examine the growth of the Levazoot 15 strain (*C. norvegensis*) with different sources of energy, nitrogen, vitamins, and microminerals.

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Materials and methods

The *C. norvegensis* (strain Levazoot 15) was from the yeast collection (GenBank accession no. JQ519367.1) of the Faculty of Animal Science and Ecology of the Autonomous University of Chihuahua, Mexico. *Candida norvegensis* was

kept viable by periodic passes by streaking a culture aliquot onto malt extract agar (DIFCO, Sparks, MD, USA), incubation at 30 °C for 30 h, followed by refrigeration storage at 4 °C. To evaluate the strain's growth in the presence of energy and nitrogen sources, non-rumen fluid (NRF) medium (Caldwell and Bryant, [12]) as modified by Elías [13], as the malt extract agar medium normally used for mold and yeast cultures, is not formulated with the specific mineral solutions required by this yeast strain [14]. From the components of the Elías [13], NRF medium were removed cellobiose, soluble starch, sodium lactate, hemin, volatile fatty acids, cysteine hydrochloride, $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$, and Na_2HCO_3 , because they are commonly used for the culture of ruminal microorganisms. Table 1 shows the composition of the medium base used.

Experimental procedure, treatment, and design

A fully randomized factorial design was used to study the nutritional requirements of *C. norvegensis* yeast, sequentially testing the following factors in four experiments: (i) energy sources: Glucose (Faga Lab, Guamuchil, Sin. Mexico), Lactose (Merck, Kenilworth, NJ, USA), Sucrose (J.T. Baker, Xalostoc, Mexico), (ii) nitrogen sources: ammonium sulfate (J.T. Baker, Xalostoc, Mexico), tryptone (DIFCO, Sparks, MD, USA), hydrolyzed casein (Sigma Aldrich, St. Louis MO, USA), urea (J.T. Baker, Xalostoc, Mexico), (iii) B-complex vitamins: thiamine (Sigma Aldrich, St. Louis MO, USA), calcium pantothenate (Sigma Aldrich, St. Louis MO, USA), niacinamide (Sigma Aldrich, St. Louis MO, USA), riboflavin (Faga Lab, Guamuchil, Sin. Mexico), pyridoxine ((Sigma Aldrich, St. Louis MO, USA), para-amino-benzoic acid (PABA) (Sigma Aldrich, St. Louis MO, USA), biotin (Faga Lab, Guamuchil, Sin. Mexico), dithionic acid (Sigma Aldrich, St. Louis MO, USA), folic acid (Sigma Aldrich, St. Louis MO, USA), cobalamin (Faga Lab,

Guamuchil, Sin. Mexico), and (iv) microminerals: copper (J.T. Baker, Xalostoc, Mexico), iron (J.T. Baker, Xalostoc, Mexico), manganese (J.T. Baker, Xalostoc, Mexico). This group of factors together was defined as factor A, which varies by type and level across the experiments. The common factor in the four sources was time, designated as factor B and consisting of measurements of the response variables at 0, 20, and 40 h (Table 2). The experiment was ran once with four replicates for each sampling time. Glucose as an energy source was replaced by sucrose and lactose. Ammonium sulfate as a nitrogen source was removed from mineral solution B and was replaced by tryptone (144 mg), casein (144 mg), and urea (24.4 mg). One milliliter of vitamin and mineral solutions were added to final concentrations of 0.002%, respectively. Growth experiment with each medium component formulation giving the best results was used in subsequent experiments that resulted in the yeast growth base medium for these series of experiments. Optical density (OD) was measured by colorimetry using a HACH DR 5000 spectrophotometer at 530-nm wavelength [15].

Preparation of yeast inoculate

A sample of the culture medium of the strain Levazoot 15 with 24 h of growth were collected and dissolved in 10 ml of malt extract broth (DIFCO St Louis MO, USA) and incubated in a 20-mL essay test tube at 30 °C for 24 h for the preparation of the pre-inoculum. Fifty milliliters of this culture was used to inoculate 50 mL of malt extract broth in a sterile 100-mL Erlenmeyer flask and was incubated as indicated above. The resulting culture was used as inoculum for all experiments. Thirty-six sterile 200-mL crystal flasks with screw caps were used for experiments examining the carbohydrate energy sources and 48 sterile 200-mL crystal flasks with screw caps were used for experiments examining the nitrogen sources. Each crystal flask with 50 ml of the modified NRF medium was inoculated with 0.5 mL of the inoculum.

Statistical analysis

Statistical analysis was performed using the GLM procedure of the SAS statistical package [16]. Comparison of means was performed using contrasts procedures and the differences were determined to be statistically significant at $p < 0.05$ or as indicated.

Results

The OD results for the *C. norvegensis* culture showed significant interactions between the various energy sources and fermentation times ($P < 0.05$; Table 3). We determined that OD increased through 40 h for all three sources, with higher values observed in the presence of glucose relative to lactose and sucrose at 20 and 40 h.

Table 1 Non-rumen fluid base medium used to determine *Candida norvegensis* (strain Levazoot 15) energy and nitrogen requirements

Ingredient	Quantity
Mineral solution A ¹	15 ml
Mineral solution B ²	15 ml
Glucose	0.4 g
Yeast extract	0.25 g
$\text{Cu}_2\text{SO}_4\cdot 5\text{H}_2\text{O}$ (0.15%, w/v ¹)	1 ml
$\text{MnCl}_2\cdot 4\text{H}_2\text{O}$ (0.1%, w/v ¹)	1 ml
CoCl_2 (0.1%, w/v ¹)	0.1 ml
Distilled water	67 ml
Total	100 ml

¹ Mineral solution A (1 L) contains 3.0 g HK_2PO_4

² Mineral solution B (1 L) contains 3.0 g H_2KPO_4 , 6.0 g $(\text{NH}_4)_2\text{SO}_4$, 6.0 g NaCl , 0.6 g $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, and 0.6 g CaCl_2

Table 2 Carbohydrate energy sources, nitrogen sources, B-complex vitamins, and micro minerals used to evaluate *Candida norvegensis* (strain Levazoot 15) growth

Experiment	Factorial pxq	Factor A*	q levels of A
i	3×3	Sources of energy	Glucose (0.4 g/100 mL) Lactose (0.4 g/100 mL) Sucrose (0.4 g/100 mL)
ii	4×3	Sources of nitrogen	Ammonium sulfate[(NH ₄) ₂ SO ₄] (0.6 g/100 mL) Tryptone (DIFCO) (144 mg/100 mL) Hydrolyzed casein (144 mg/100 mL) Urea (24.4 mg/100 mL)
iii	12×3	B-complex vitamins	No vitamin All vitamins All except thiamine (20 mg/100 mL) All except calcium pantothenate (20 mg/100 mL) All except niacinamide (20 mg/100 mL) All except riboflavin (20 mg/100 mL) All except pyridoxine (20 mg/100 mL) All except PABA (1 mg/100 mL) All except biotin (0.5 mg/100 mL) All except dithionic acid (0.5 mg/100 mL) All except folic acid (0.5 mg/100 mL) All except cobalamin (0.2 mg/100 mL)
iv	5×3	Microminerals	No trace element All trace elements Copper (3.0 ppm) Iron (6.82 ppm) Manganese (2.64 ppm)

*Only variations in levels of A are included; variation in B was the time factor, with $q = 3$ levels (0, 20, and 40 h)

The OD results for *C. norvegensis* culture also showed significant interactions between nitrogen sources and fermentation times ($P < 0.05$; Table 3). However, the highest value was seen at 40 h with tryptone serving as the nitrogen source ($P < 0.05$).

The results of this study show that *C. norvegensis* (strain Levazoot 15) does not require the addition of

B-complex vitamins for optimal growth. No difference was observed between media with no vitamin and all of the vitamins used in this study (Table 4).

Growth results showed the interaction between the type of mineral and fermentation time (Table 4). The highest growth rates in the yeast strain occurred at 40 h in the presence of all minerals and Mn ($P < 0.05$).

Table 3 Optical density means ($\pm$ SD) of *Candida norvegensis* (strain Levazoot 15) cultured over time with different carbohydrate energy sources and protein sources

Treatment	Sampling time point (h)			SE
	0	20	40	
Glucose (0.4 g/100 mL)	0.036 ^{cd} (± 0.005)	1.477 ^{bd} (± 0.025)	1.693 ^{ad} (± 0.012)	0.0062
Lactose (0.4 g/100 mL)	0.054 ^{cd} (± 0.004)	0.331 ^{bc} (± 0.011)	0.341 ^{af} (± 0.008)	0.0062
Sucrose (0.4 g/100 mL)	0.050 ^{bd} (± 0.000)	0.416 ^{ac} (± 0.008)	0.471 ^{ac} (± 0.003)	0.0062
Ammonium sulfate (0.6 g/100 mL)	0.051 ^{cd} (± 0.008)	0.084 ^{bc} (± 0.004)	0.126 ^{af} (± 0.003)	0.003
Tryptone (144 mg/100 mL)	0.054 ^{cd} (± 0.0008)	0.169 ^{bd} (± 0.005)	0.199 ^{ad} (± 0.016)	0.003
Hydrolyzed casein (144 mg/100 mL)	0.053 ^{cd} (± 0.0005)	0.163 ^{bd} (± 0.005)	0.183 ^{ac} (± 0.005)	0.003
Urea (24.4 mg/100 mL)	0.049 ^{cd} (± 0.000)	0.1 ^{bc} (± 0.009)	0.142 ^{af} (± 0.009)	0.003

^{abc} Superscripts refer to row mean comparisons between time

^{def} Superscripts refer to column mean comparisons between sugar sources and between nitrogen sources

$n = 9$ for sugar sources and $n = 12$ for nitrogen sources; SE, standard error; $\pm$ = range of the standard deviation

Table 4 Optical Density means ($\pm$ SD) of *Candida norvegensis* (strain Levazoot 15) cultured over time with different B-complex vitamins and microminerals

Treatment	Sampling time point (h)			SE
	0	20	40	
All vitamin	0.0592 ^{bf} ($\pm$ 0.003)	0.071 ^{bc} ($\pm$ 0.008)	0.1304 ^{ad} ($\pm$ 0.011)	0.0064
No vitamin	0.071 ^{bd} ($\pm$ 0.001)	0.0792 ^{be} ($\pm$ 0.003)	0.1266 ^{ad} ($\pm$ 0.014)	0.0064
Thiamin (20 mg/100 mL)	0.0674 ^{be} ($\pm$ 0.005)	0.0712 ^{be} ($\pm$ 0.002)	0.1246 ^{ae} ($\pm$ 0.015)	0.0064
Calcium panth (20 mg/100 mL)	0.0712 ^{ad} ($\pm$ 0.002)	0.0814 ^{ae} ($\pm$ 0.006)	0.1034 ^{af} ($\pm$ 0.005)	0.0064
Niacinamide (20 mg/100 mL)	0.070 ^{bd} ($\pm$ 0.002)	0.0762 ^{be} ($\pm$ 0.002)	0.1158 ^{af} ($\pm$ 0.023)	0.0064
Riboflavin (20 mg/100 mL)	0.0724 ^{bd} ($\pm$ 0.003)	0.0826 ^{be} ($\pm$ 0.007)	0.1482 ^{ad} ($\pm$ 0.017)	0.0064
Pyridoxine (20 mg/100 mL)	0.0564 ^{bf} ($\pm$ 0.002)	0.0780 ^{abe} ($\pm$ 0.006)	0.1058 ^{af} ($\pm$ 0.010)	0.0064
PABA (1 mg/100 mL)	0.0544 ^{bf} ($\pm$ 0.0005)	0.0838 ^{be} ($\pm$ 0.016)	0.132 ^{ad} ($\pm$ 0.029)	0.0064
Biotin (0.5 mg/100 mL)	0.0598 ^{bf} ($\pm$ 0.003)	0.0794 ^{be} ($\pm$ 0.013)	0.1456 ^{ad} ($\pm$ 0.011)	0.0064
Dithionic acid (0.5 mg/100 mL)	0.0714 ^{bd} ($\pm$ 0.0009)	0.0982 ^{bd} ($\pm$ 0.006)	0.1548 ^{ad} ($\pm$ 0.043)	0.0064
Folic acid (0.5 mg/100 mL)	0.0712 ^{bd} ($\pm$ 0.023)	0.1032 ^{bd} ($\pm$ 0.011)	0.1530 ^{ad} ($\pm$ 0.031)	0.0064
Cobalamin (0.2 mg/100 mL)	0.0708 ^{bd} ($\pm$ 0.002)	0.0900 ^{bde} ($\pm$ 0.017)	0.1608 ^{bd} ($\pm$ 0.031)	0.0064
All mineral	0.0076 ^{bd} ($\pm$ 0.002)	0.0074 ^{bd} ($\pm$ 0.004)	0.0670 ^{ad} ($\pm$ 0.007)	0.0024
No mineral	0.008 ^{bd} ($\pm$ 0.0007)	0.006 ^{bd} ($\pm$ 0.003)	0.0224 ^{af} ($\pm$ 0.001)	0.0024
Copper (3.0 ppm)	0.007 ^{bd} ($\pm$ 0.001)	0.0086 ^{bd} ($\pm$ 0.004)	0.0346 ^{ae} ($\pm$ 0.007)	0.0024
Iron (6.82 ppm)	0.0032 ^{bd} ($\pm$ 0.001)	0.0110 ^{bd} ($\pm$ 0.0007)	0.023 ^{ae} ($\pm$ 0.002)	0.0024
Manganese (2.64 ppm)	0.0078 ^{bd} ($\pm$ 0.003)	0.0104 ^{bd} ($\pm$ 0.005)	0.0558 ^{ad} ($\pm$ 0.016)	0.0024

^{abc} Superscripts refer to row mean comparisons between time^{def} Superscripts refer to column mean comparisons between vitamins sources and between minerals sources $n = 36$ for vitamins sources and $n = 15$ for minerals sourcesSE; standard error; $\pm$ = range of the standard deviation

Discussion

Since Levazoot 15 strain of *Candida norvegensis* has shown promise as a rumen fermentation activator *in vitro* and *in situ*, creating a better ruminal environment in many ways, those findings suggest a need to study the growth requirements of this yeast strain, for development of an economical culture medium that will lead to large-scale biomass production. Culture optical density measurements are known to be linked closely to greater microbial development and can be an indirect measure of growth [17]. Thus, it can be deduced that the *C. norvegensis* (strain Levazoot 15) utilized all three carbohydrate sources examined in this study, but achieved greater growth in the presence of glucose. Kurtzman and Robnett [18] described the capability of yeasts to use a wide array of compounds, including carbohydrates, which renders them quite valuable in industry and agriculture. Cárdenas [19] and Sandven et al. [20] obtained similar results, showing that *C. norvegensis* ferments glucose, but not sucrose or lactose. Although sugar fermentation and digestion capabilities of yeasts are closely related, all sugars can be digested by yeasts; however, some sugars can be digested by oxidation but not by fermentation [11].

The strain study grew in the presence of all four sources of nitrogen through to 40 h, confirming that yeast can utilize nitrogen in a variety of forms, especially ammonium sulfate, which is

also a source of sulfur for synthesis of some amino acids, although urea, peptone, and other soluble protein derivatives can also be used [11, 21]. Tryptone is a pancreatic digest of casein that is used as a nitrogen source in culture medium. It contains a large amount of tryptophan, an essential amino acid that microorganisms use to produce indole, pyruvic acid, and ammonia. For this reason, we can deduce that the *C. norvegensis* (strain Levazoot 15) selectively utilized nitrogen sources.

According to Villamil and Zapata [11], only some yeasts require B-complex vitamins (e.g., thiamine, biotin, inositol, pantothenic acid) for growth. This observation is in agreement with the results of our experiments, as we observed no statistical difference in OD with the inclusion of each of these vitamins.

Unlike vitamins, microminerals are required by the yeast strain for optimal growth. Meinander and Hahn-Hägerdal [22] proposed that microorganisms generally grow more vigorously in glucose-rich medium than in mineral-rich medium, as energy-rich medium can be processed directly via anabolic pathways, reducing the need for biosynthesis precursors and saving metabolic energy. However, use of glucose-rich medium may result in the unexpected loss of plasmids and even cause chromosomal changes in the microorganisms. For example, in prolonged culture, such as in continuous fermentation, the probability of harmful genetic instability is high. Hahn-Hägerdal et al. [23] evaluated the effects of four culture

media commonly used for yeast growth under limited aerobic and oxygen conditions, and that of a mineral media designed to overcome growth limitations due to high concentrations of vitamins and oligo-elements. They found that sodium chloride, riboflavin, and folic acid were not necessary for *S. cerevisiae* growth, whereas cobalt stimulated growth. Their work supports the concept that complex medium components can mask inherent limitations in recombinant strain metabolisms, as demonstrated by *Escherichia coli* and *S. cerevisiae* [24, 25]. In addition, all organisms are known to require iron, which acts as a cofactor with enzymes involved in a range of biological processes, such as cellular respiration and the synthesis of metabolic intermediates [26].

The results of this study contribute to our knowledge of the nutritional requirements of *C. norvegensis* (strain Levazoot 15), which can aid the preparation of culture medium with local substrates as yeast-based additives that promote ruminal activation and are affordable for livestock producers.

Based on the findings of this study, we can conclude that the yeast *C. norvegensis* (strain Levazoot 15) preferentially utilizes glucose as a source of energy, tryptone as a source of nitrogen, and manganese as a mineral source, and that no vitamin source is necessary for growth.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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