REVISTA DE LA SOCIEDAD VENEZOLANA DE MICROBIOLOGIA

RSVM

Revista de la Sociedad Venezolana de Microbiología 2014; 34:38-42

Artículo original

Nutritional requirements of a *Saccharomyces cerevisiae* starter culture used in the elaboration of wine from orange

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Recibido 13 de diciembre de 2013; aceptado 9 de abril de 2014

***Abstract*:** The study of the yeasts present in the orange juice microbiota and used in the winemaking involves the isolation, identification and production of biomass. In order to optimize the yeast growth, the nutritional requirements should be determined. The aim of this work was to determine the vitamin and other growth organic factors demand for the *S. cerevisiae* isolated from fermented orange juice. The culture medium was formulated with sucrose (4 g/L) as carbon and energy source (CES), urea as nitrogen source (NS), six vitamins (calcium pantothenate, pyridoxine, thiamine, biotin, niacin, folic acid), and inositol and *p*-aminobenzoic acid (PABA) as growth factors. The complete culture medium was used as blank, and three other media were prepared: without the vitamins and growth organic factors, with calcium pantothenate, pyridoxine, thiamine, biotin and inositol and with the latter four vitamins, but for inositol. Finally, four new media were obtained by elimination of one vitamin at a time. The temperature was set at 30 ºC and the pH, at 5.0. The *S. cerevisiae* present in the orange juice needs calcium pantothenate, pyridoxine, thiamine and biotin to grow adequately under aerobic conditions in a batch system, not being auxotroph for niacin, folic acid, PABA and inositol.

***Keywords*:** *Saccharomyces cerevisiae,* orange wine, nutritional requirements.

Requerimientos nutricionales de un cultivo iniciador de *Saccharomyces cerevisiae* utilizado en la elaboración de vino de naranja

***Resumen*:** El estudio de levaduras autóctonas para ser usadas en la elaboración de vino de naranja, incluye aislamiento, identificación y obtención de biomasa. Para optimizar su crecimiento se deben establecer los requerimientos nutricionales. El objetivo de este estudio fue definir las necesidades de vitaminas y factores orgánicos de crecimiento de *S. cerevisiae* aislada de jugo de naranja fermentado. Se formuló un medio de cultivo con sacarosa (4 g/L) como fuente de carbono y energia (FCE) y urea como fuente de nitrógeno (FN), con 6 vitaminas (pantotenato de calcio, piridoxina, tiamina, biotina, niacina, ác. fólico) e inositol y ácido *p*-aminobenzoico (PABA) como factores de crecimiento. El medio completo se usó como testigo, preparándose además otros tres medios: con ausencia de todas las vitaminas y factores orgánicos de crecimiento, con pantotenato de Ca, piridoxina, tiamina, biotina e inositol y con esas 4 vitaminas, pero sin inositol. Finalmente, se procedió a eliminar una vitamina. Los cultivos se realizaron en aerobiosis a 30 ºC y pH 5,0. La cepa autóctona de *S. cerevisiae* requiere las vitaminas pantotenato de calcio, piridoxina, tiamina y biotina para crecer adecuadamente en condiciones aeróbicas en sistema batch; en cambio, no es auxótrofa para niacina, ácido fólico, PABA e inositol.

***Palabras clave*:** *Saccharomyces cerevisiae,* vino de naranja, requerimientos nutricionales.

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Introduction

The transformation of must into wine is a spontaneous process carried out by yeasts, *Saccharomyces cerevisiae* species in particular, whose origin has become a controversial issue. Though traditionally all the species involved in wine fermentation have been assumed to be present in the grapefruit surface, some further studies reported the presence of only non-*Saccharomyces* species, *S. cerevisiae* being found mainly in wine cellar environments [1-3].

At present, wine manufacturing processes tend to use starter cultures which make fermentation faster and predictable. In order to obtain high quality, nice wines having no sensory defects, it is important to know the role

yeasts play in fermentation. Although a great variety of wine yeasts can be found in the market, there has been no agreement between the results obtained from their use. It is for this reason that autochthon strains are being selected to be used as starters, these strains being easier to adapt to the musts and wines with specific characteristics being obtained [4,5].

Prior to their selection, native yeasts require the isolation and identification of the yeasts associated to spontaneous fermentation processes, this selection being carried out on the basis of the evaluation of certain technological properties which show their potential capacity for enological use [6,7].

The study of the yeasts present in the microbiota of orange juice and used in the winemaking from oranges involves the isolation, identification and production of biomass, from where the inoculum for wine fermentation is prepared [8].

In order to optimize the yeast growth, the nutritional requirements of the strain to be used should be determined [9].

For fungi development, small quantities of several organic compounds are necessary, these compounds being neither the nitrogen or the carbon and energy sources (NS and CES, respectively) nor the inorganic compounds, which are needed in higher quantities. They are the so-called growth factors and include the vitamins and other organic molecules taking part in the microbial metabolism [10].

On the one hand, vitamins encourage the inoculum growth, in concentrations between 0.01 and 1.0 ppm, and also play catalytic functions as coenzymes or enzyme constituents. On the other hand, a group of organic compounds, characterized as non-vitamins, play an active role at low concentrations (about 10 ppm), fatty acids being included among them [11].

These compounds can be synthesized from simple precursors by organisms called auxo-autotrophs, which differ from those called auxo-heterotrophs, since the latter either do not synthesize some of these substances or their production does not meet the actual needs, which in turn makes necessary the further administration of these compounds to the culture medium [12].

The aim of the present work was to determine the demand of vitamin and other growth organic factors for the *S. cerevisiae* isolated from fermented orange juice. A base medium CES-limited was used so as to formulate a complete culture medium from where to obtain biomass, which was then used as inoculum in the elaboration of wine from oranges.

Materials and methods

Formulation of the culture medium: The culture medium was formulated with sucrose (4 g/L) as carbon and energy source (CES), urea as nitrogen source (NS), six vitamins (calcium pantothenate, pyridoxine, thiamine, biotin, niacin, folic acid), and inositol and p-aminobenzoic acid (PABA) as growth factors [13]. The pH was set at 5.0. CES is the limitant substrate and it was added in a low enough concentration in order to reducing the Crabtree effect, and then get a better yield. Table 1 shows the base broth composition.

Table 1. Composition of the base medium for the determination of a *Saccharomyces cerevisiae* culture nutritional requirements.

Components	Quantities (g/L)
Sucrose	4
Urea	1.3 (3.2 mL/L mother solution*)
$NaH_2PO_4 \cdot H_2O$	0.8
Microelements	1 mL stock solution 1
$MgCl_2 6 H_2O$	0.6
KCl	0.4
Na_2SO_4	0.5
$CaCl_2 \cdot 2H_2O$	0.1
Vitamins	1 mL stock solution 2
Composition of stock solution 1	
$FeSO_4 \cdot 7H_2O$	15
$ZnSO_4 \cdot 7H_2O$	5
$MnSO_4 \cdot H_2O$	3
$CuSO_4 \cdot 5H_2O$	0.7
$CoCl_2 \cdot 6H_2O$	0.2
$Na_2MoO_4 \cdot H_2O$	0.6
H_3BO_3	0.1
KI	0.1
Citric acid	50
Composition of stock solution 2	
Niacin	12
Calcium Pantothenate	4
Pyridoxine	1
Thiamine –HCl	1
Folic acid	1
PABA	1
Biotin	0.06
m-Inositol	60

* 400 g/L of urea.

Sterilization of medium components: The sterilization of both CES and salts (except phosphate) was carried out at 121 °C, during 15 minutes. However, phosphate solution sterilization was carried out in isolation following the procedure mentioned above, in order to prevent the interaction with divalent ions and hence the formation of

insoluble precipitates, which cannot be assimilated by the microorganism. Because of their thermolability, the urea and vitamin stock solutions were sterilized by filtration, filters of 0.45µm and 0.22 µm pore size being used.

The different solutions were mixed under aseptic conditions before starting the inoculations. Table 2 shows the combinations of the vitamins added to the base medium.

The complete culture medium was used as blank (1), three other media being prepared from the same base: without the addition of all the vitamins and growth organic factors (2), with the addition of calcium pantothenate, pyridoxine, thiamine, biotin and inositol (3) and with the addition of the latter four vitamins, but for inositol (4).

Table 2. Combination of vitamins and growth factors added to the base medium for *S. cerevisiae* culture.

Vitamin	1	2	3	4
Niacin	X			
Calcium Pantothenate	X		X	X
Pyridoxine	X		X	X
Thiamine	X		X	X
Folic acid	X			
PABA	X			
Biotin	X		X	X
Inositol	X		X	

Inoculation and growth of S. cerevisiae in the different media: The media described in table 2 were used to study *S. cerevisiae* requirements for vitamins and growth factors. Experiments where vitamins and growth factors were progressively eliminated were carried out, followed by an observation of the microorganism behavior (growth or absence of growth) as a result of the successive component eliminations and a series of flask transfers. The procedure used may be described as follows: all four different media (one "complete", having all the components, and the others lacking the addition of some of them) were inoculated with the yeast under study, which was isolated from a streak culture prepared in yeast glucose chloramphenicol (YGC) agar and then diluted with sterile water until an initial OD_{625} of 0.2 was reached. Each inoculated medium (100 mL), contained in conical flasks of 1000 mL, was incubated at 30 °C for 24 hours in shaker at 200 rpm. The growth of the different culture media was studied by determining the pH, OD_{625}, concentration of the remaining sucrose (Somogyi-Nelson technique preceded by an inversion with HCl 0.1 N), dry mass (DM) and microscopic count in a Neubaüer chamber.

After 24 hours of incubation, a variable volume was taken from each flask and transferred, under aseptic conditions, to a second conical flask, the variable volume resulting from the need to have the same number of microorganisms in each of the different media contained in the new four flasks. The same procedure was repeated for every medium four times at the most, depending on the presence or absence of microorganism growth.

Medium 4, that lacks niacin, folic acid, PABA and inositol, with an OD_{625}=5,346, was used to inoculate four new flasks containing media without pyridoxine (4a), calcium pantothenate (4b), thiamine (4c) and biotin (4d) respectively. These new media being then inoculated with samples taken from medium 4 and treated following the procedure described above.

Results and discussion

S. cerevisiae growth in culture media having different vitamin availability: Figure 1 shows pH, OD_{625}, concentration of the remaining sucrose and DM in the media 1, 2, 3 and 4. The values obtained after 24 hours of incubation were considered as the initial ones, a first and second transfer being carried out every 24 hour

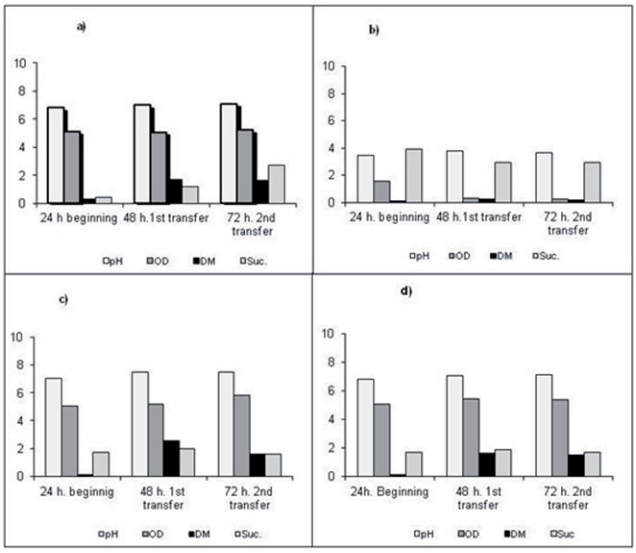

Figure 1. Evolution of *S. cerevisiae* growth parameters in media with different vitamin availability: a) complete medium , b) vitamin-free medium, c) medium without the addition of niacin, thiamine and folic acid, and d) medium without the addition of niacin, thiamine, folic acid and inositol.

OD_{625} evolution: After 24 hours, a slight increase was observed (OD_{625}= 1.512) in the medium without vitamin addition (Figure 1b). Similar values (OD_{625}~ 5.1) were obtained in the case of the other three media (Figures 1a, c and d).

After carrying out the first transfer, OD_{625} increase was even lower (OD_{625}= 0.3) (Figure 1b) for medium 2, whereas it remained constant for the other three media. These results were confirmed by those obtained after the second transfer.

pH evolution: For the complete medium (1) the pH showed a value closed to neutrality, both in the initial medium and in the first and second transfer (Figure 1a). In the case of media 3 and 4, similar values were obtained (Figures 1c and d). However, medium 2 showed an acidification, final pH

being between 3.4 and 3.8 (Figure 1b).

Dry mass evolution: While media 1, 3 and 4 showed a 10% increase in DM (1.5 g/L), no variations were observed in medium 2, values being close to 0.3 g/L (Figures 1a, b, c and d). The remaining sucrose in media 1, 3 and 4 was in the order of mg/L (Figures 1a, b and d). However, for medium 2, sucrose was practically not metabolized, the remaining being 2.9 g/L (Figure 1b).

Microscopic count: While media 1, 3 and 4 showed a microscopic recount in the order of 10^8 cells/mL, medium 2 showed values of 10^6 cells/mL, which *a priori* indicates that some of the vitamins and growth factors studied are essential to yeast development.

In order to demonstrate which of them are necessary, from the medium having no niacin, folic acid, PABA or inositol (4), new media were prepared. This was due to the fact that, as table 3 shows, the results corresponding to medium 4 are similar to those obtained for the complete medium (1).

Table 3. Final results for *S. cerevisiae* in the media 1, 2, 3 and 4 after two transfers each, and in media resulting from medium 4 with different vitamin composition

Medium	pH	OD_{625}	DM (g/L)	Sucrose (mg/L)	$Y_{X/S}$	NºYeasts/mL
1	7.10	5.226	1.64	27	0.36	1.4×10^8
2	3.78	0.276	0.2	2930		5.0×10^5
3	7.49	5.808	1.62	16	0.35	1.3×10^8
4	7.15	5.346	1.51	17	0.38	1.4×10^8
4a	3.59	2.200	0.53	9	0.13	-
4b	7.81	3.450	0.93	11	0.23	-
4c	7.90	3.435	0.86	7	0.21	-
4d	3.29	2.445	0.52	19	0.13	-

For the media 1, 3 and 4, biomass performance ($Y_{X/S}$,), with respect to the limiting substrate, was about 0.4, ethanol being possibly obtained (Crabtree effect).

From medium 4, whose OD_{625}= 5.346 and lacks the addition of niacin, folic acid, PABA or inositol , four new flasks containing media without the addition of calcium pantothenate (4a), pyridoxine (4b), thiamine (4c) and biotin (4d) were inoculated. Table 3 shows these results.

Results obtained after 24 hours were compared, a decrease of pH (pH=6.7-4.4) being observed in every case (Figures 2a, b, c and d).

In general, the increase was much lower than in the previous series, a low OD_{625} being observed, similar for the four media. Values varied within a range of 2, being lower in the case of the medium lacking biotin (OD_{625}= 1.952) (Figure 2d).

CES consumption was almost complete, there being a remaining which ranged between 0.7 mg/mL, for the medium without pyridoxine, and 21 mg/mL for the medium without thiamine (Figures 2b and c). However, after 48 hours, a consumption decrease was observed for the medium lacking biotin, the other media not showing this decrease.

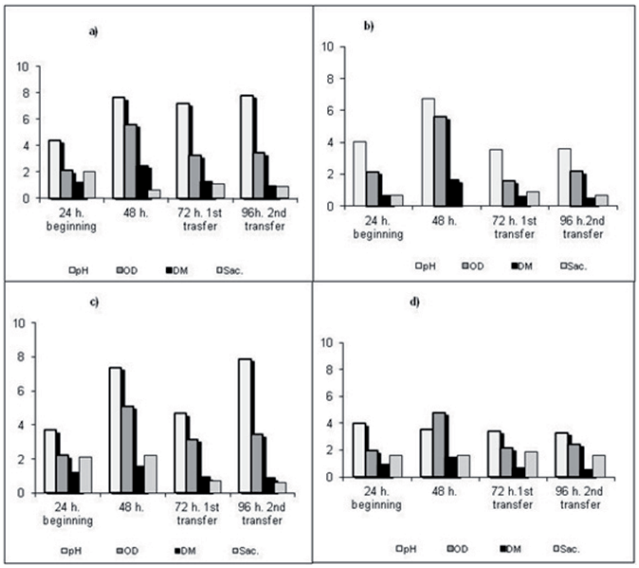

Figure 2. Evolution of the growth parameters in the different media resulting from medium 4: 4a) without pyridoxine, 4b) without calcium pantothenate, 4c) without thiamine and 4d) without biotin.

Figure 2 shows media alkalinization if compared to the pH obtained after 24 hours, pH ranging between 6.7 and 7.6, except for the medium lacking biotin, which acidified, and the pH decreased from 4 (at 24 hs) to 3.5 (at 48 hours).

OD_{625} increased in every case, this increase being lower for the medium without biotin.

The remaining sucrose for the media both without calcium pantothenate and pyridoxine was close to zero. However, for the media both without thiamine and biotin the values showed no variation compared to those obtained after 24 hours.

For the first and second transfer, a decrease (in the remaining sucrose) was observed when the medium lacked calcium pantothenate (Figure 2b). On the other hand, though the media lacking pyridoxine and thiamine showed a different increase compared to that corresponding to the blank having all the vitamins, even in the second transfer, OD_{625} reached the values 3.45 and 3.43, respectively. These are even higher than those recorded for media lacking calcium pantothenate (2.2) and biotin (2.44) (Fig. 2a, b, c and d) [13,14].

It is important to point out that *S. cerevisiae* requirements for biotin are very significant since urea has been used as NS, which makes necessary for the microorganism to synthesize three enzymatic systems containing biotin in order to grow under aerobic conditions [15].

Under the absence of calcium pantothenate and biotin, the yeast acidified the medium and reached a $Y_{X/S} \cong 0.1$; probably due to the fact that part of the CES was used to produce organic acids which could not be assimilated afterwards. This in turn led to a lower biomass performance [16].

Under the absence of pyridoxine and thiamine, $Y_{X/S} \cong 0.2$, and a final alkalinization of the culture medium (Table 3.) occurred, which could be due to the formation of organic acids, which were then assimilated, though without reaching the biomass performances of the blank medium [17].

Finally, it must be noted that in these assays both DM and OD_{625} were carried out in order to evaluate the microbial growth, every medium showing a good correlation between both parameters (Figure 3).

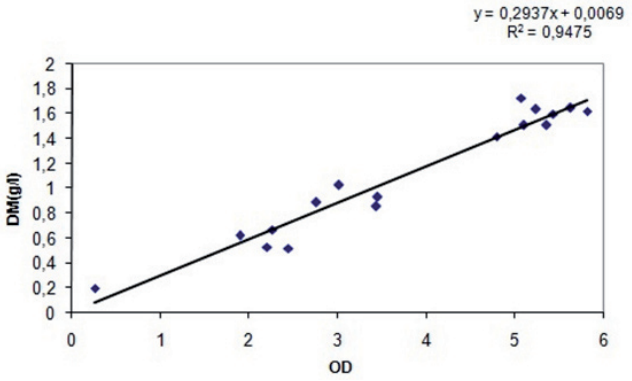

Figure 3. Relationship between OD_{625} and DM during Saccharomyces cerevisiae's growth in all cultures assayed.

Conclusions

The *S. cerevisiae* present in the orange juice needs the vitamins assayed (calcium pantothenate, pyridoxine, thiamine and biotin) to grow adequately under aerobic conditions in a batch system, not being auxotroph for niacin, folic acid, PABA and inositol.

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