EFFECT OF SOME VITAMINS AND MICRONUTRIENT DEFICIENCIES ON THE PRODUCTION OF HIGHER ALCOHOLS BY *Saccharomyces cerevisiae*.

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ABSTRACT: A study was carried out in order to determine the effect of vitamins (biotin, thiamine, pantothenic acid and pyridoxal) and micronutrient (zinc, boron, manganese and iron) deficiencies on higher alcohol production during alcoholic fermentation with the industrially used yeast *Saccharomyces cerevisiae* M-300-A. Zinc deficiency induced a reduction on the levels of isobutyl and isoamyl alcohols. An increase on isobutyl alcohol (fivefold) and a reduction of isoamyl alcohol (two fold) and n-propyl alcohol (three fold) contents resulted from pantothenic acid deficiency, whereas pyridoxal deficiency caused an increase on the levels of isobutyl and isoamyl alcohols. Biotin was not essential for the growth of this strain.
Key Words: vitamins, *Saccharomyces*, yeasts, micronutrient, higher alcohols, alcoholic fermentation

EFEITO DA DEFICIÊNCIA DE VITAMINAS E MICRONUTRIENTES SOBRE A PRODUÇÃO DE ÁLCOOIS SUPERIORES POR *Saccharomyces cerevisiae*

RESUMO: Foi estudado o efeito da deficiência das vitaminas (biotina, tiamina, ácido pantotênico e piridoxal) e de micronutrientes (boro, zinco, manganês e ferro) sobre a produção de álcoois superiores durante a fermentação alcoólica com a levedura industrial *Saccharomyces cerevisiae* M-300-A. Com a deficiência de zinco ocorreu redução na formação dos álcoois isobutílico e isoamílico enquanto que a deficiência de pantotenato provocou aumento no nível de álcool isobutílico (cerca de cinco vezes) e redução dos álcoois isoamílico (duas vezes) e n-propílico (tres vezes). Na deficiência de piridoxina ocorreu aumento nos teores de isobutílico e de isoamílico. A biotina não foi essencial para o crescimento dessa linhagem de levedura.
Descritores: vitaminas, *Saccharomyces*, levedura, micronutrientes, álcoois superiores, fermentação alcoólica

INTRODUCTION

Higher alcohols and their esters are important components of the flavour and aroma of alcoholic fermented beverages (SUOMALAINEN, 1971; SUOMALAINEN & LEHTONEN, 1979). The mixture of higher alcohols, acids and esters, generally referred to as fusel oil, is of considerable importance on the aroma of beers (SIHTO & ARKIMA, 1963). Isoamyl alcohol (3-methyl-1-butanol) constitutes the highest proportion of the alcohol mixture with smaller amounts of n-propyl alcohol (WEBB & INGRAHAM, 1963).

Higher alcohols are formed as by-products in alcoholic fermentation carried out by yeasts (WEBB & INGRAHAM, 1963) and bacteria (BEVERS & VERACHTERT, 1976) from decarboxylation of ketoacid intermediates in the leucine, isoleucine, valine and threonine biosynthesis (INGRAHAM & GUYMON, 1960; SUOMALAINEN & KAHANPAA, 1963; YLANEN, 1966). The addition of these amino acids to a fermentation medium may increase the total formation of the corresponding higher alcohols, although 50% or more of the fusel alcohols may arise in a beer wort from carbohydrates (SCHULTHESS & ETTLINGER, 1978).

There are several known factors affecting higher alcohol production by yeasts such as temperature (HOUGH & STEVENS, 1961), yeast species (FAHRASMANE et al., 1985), aeration (WEBB & INGRAHAM, 1963), magnesium (NORDSTROM & CARLSSON, 1965; GILDENHUYS & SLAUGHTER, 1983) and nitrogen source (AYRAPAA, 1971; BORZANI et al., 1981). The addition of yeast growth inhibitors could also lead to a reduction of higher alcohols as has been shown by NORDSTROM & CARLSSON

(1965) for 2,4-dinitrophenol, GUTIERREZ (1988) for sulphite, GUTIERREZ & ORELLI (1991) for nitrite, GUTIERREZ et al. (1991a) for benzoate and GUTIERREZ et al. (1991b) for acetic acid.

However, it is apparent that little attention has been paid to the effect of micronutrients and vitamin deficiencies on higher alcohol production during ethanolic fermentation. The following study was carried out with the aim of investigating this subject.

MATERIAL AND METHODS

YEAST. The organism used in this study was *Saccharomyces cerevisiae* M-300-A, currently in use in Brazil for the production of ethanol.

FERMENTATION MEDIUM. The medium contained per litre: sucrose, 120 g (micronutrient assay) and 140 g (vitamin assay); citric acid, 6 g; ammonium sulphate, 1.2 g; KH_2PO_4, 1.5 g; $MgSO_4$, 0.75 g; $CaCl_2.2H_2O$, 0.37 g; ergosterol, 0.005 g and oleic acid, 0.030 g. The following micronutrients were added, per litre: boric acid, 4.7 mg; zinc sulphate.$7H_2O$, 4.7 mg; aluminium sulphate.18 H_2O, 2.7 mg; cupric sulphate, $5H_2O$, 1.9 mg; manganese sulphate. H_2O, 1.3 mg; cobalt chloride, $2H_2O$, 0.4 mg; potassium iodide, 0.4 mg; ferrous sulphate, $7H_2O$, 2.3 mg. The following vitamins were added, per litre: nicotinic acid, 3 mg; biotin, 0.3mg; Ca-D-pantothenic acid, 3 mg; thiamine, 3 mg; inositol, 15 mg and pyridoxal, 3 mg. The pH was adjusted to 4.0 with 5N KOH and the media sterilized by filtration. The deficient media were obtained by omission of boron, zinc, manganese, and iron salts and thiamine, biotin, pyridoxal and D-pantothenic acid.

FERMENTATION PROCESS. Fermentations were carried out with 250 ml of medium in 500 ml pyrex conical flasks capped with aluminum foil. They were inoculated with 25 mg of yeast (dry matter basis). The flasks were incubated at 33°C and carefully shaken every two hours to ressuspend the yeast. When the sugar was completely exhausted the media were analysed for higher alcohols, ethanol and pyruvic acid contents; yeast growth was also determined. The experiments were made in triplicate and repeated twice.

ANALYSIS.

Ethanol. 25 ml of the centrifuged broth was distilled and 50 ml of the distillate collected in a Kjeldahl modified apparatus. Ethanol was estimated by densimetry according to AMORIM et al. (1979) with an Anton Paar DMA-46 densimeter.

Pyruvic acid. Pyruvic acid was determined according to FRIEDMANN & HAUGEN (1943).

Higher alcohols. Higher alcohols were analysed by direct injection of 2 microliters of the centrifuged broth in a packed column (2 m by 4.8 mm) with Hallcomid M-18 (15% wt). Analyses were performed in a CG-17 Gas Chromatograph provided with a flame ionization detector. The injector and detector temperatures were 180 and 240 °C, respectively, and the column oven operated isothermally at 110°C. Pure n-propyl, isobutyl and isoamyl alcohols were used as standards. This column does not discriminate isoamyl from active amyl alcohol, so they were both expressed as isoamyl alcohol.

YEAST GROWTH. The yeast concentration was determined gravimetrically after centrifugation followed by washing and drying at 100- 105°C.

RESULTS AND DISCUSSION

TABLE 1 shows the effect of vitamin deficiency on yeast growth and on the ethanol and pyruvic acic production during alcoholic fermentation. A higher reduction on yeast growth was observed in the pantothenic acid deficiency, confirming earlier observations of OLSON & JOHNSON (1949) and NORDSTROM (1962). A reduction of yeast growth was also observed with thiamine and pyridoxal deficiencies.

The strain used in this study did not show any special requirement for biotin.

Thiamine is essential for pyruvate decarboxylase activity (DIXON & WEBB, 1964) which could explain the higher excretion of pyruvic acid (TABLE 1) on thiamine deficiency as it has already been pointed out by LAFON-LAFOURCADE & PEYNAUD (1966).

Pantothenic acid is necessary for coenzyme A and acetyl-CoA synthesis (NORDSTROM, 1963) and, therefore, for isoamyl alcohol production (WEBB & INGRAHAM, 1963).

TABLE 1 - Effect of vitamin deficiency on yeast growth, ethanol and pyruvic acid production during alcoholic fermentation (each result is the average of six determinations ± standard error)

Treatments	Yeast growth	Ethanol	Pyruvic acid
	mg/100 ml	% volume	mg/liter
Complete	367 ± 10	8.69 ± 0,04	136 ± 12
- Pantothenic	300 ± 23	8.29 ± 0.04	257 ± 10
- Thiamine	300 ± 14	8.54 ± 0.09	474 ± 13
- Pyridoxal	307 ± 18	8.66 ± 0.05	236 ± 15
- Biotin	358 ± 9	8.69 ± 0.04	136 ± 11

TABLE 2 - Effect of vitamin deficiency on higher alcohol production (each result is the average of six determinations ± standard error) expressed in mg/litre.

Treatments	n-propryl	isobutyl	isoamyl
Complete	20 ± 2	28 ± 4	166 ± 6
- Pantothenic	7 ± 2	151 ± 9	86 ± 6
- Thiamine	39 ± 2	140 ± 6	140 ± 9
- Pyridoxal	18 ± 2	66 ± 6	230 ± 13
- Biotin	20 ± 3	21 ± 3	162 ± 5

This fact could explain the data of TABLE 2 in relation to the reduction of isoamyl alcohol and the increase of isobutyl alcohol when pantothenic acid was deficient in the medium.

The reduction of n-propyl alcohol could be due to the reduction of aspartic acid and threonine formation (WEBB & INGRAHM, 1963). However, NORDSTROM & CARLSSON (1965) did not observe a reduction of amyl alcohol with pantothenic deficiency. NORDSTROM (1964) has reported that in pantothenic acid deficiency resulted in less yeast growth with lower ethyl acetate and higher acetic acid formation.

Besides the effect on higher alcohols formation with pantothenic deficiency, a hydrogen sulphide odour was observed in the fermented medium as it had already been related by WAINWRIGHT (1962). These data show the role of pantothenic acid in the media for the production of alcoholic beverages.

Although thiamine is necessary for pyruvate decarboxylase activity (DIXON & WEBB, 1964), there was an increase in n-propyl and isobutyl alcohols and a reduction of isoamyl alcohol (TABLE 2). But as this vitamin is also necessary for the synthesis of pantothenic acid (WHITE et al., 1964), this can explain the reduction in isoamyl alcohol content.

GUTIERREZ (1988) related a reduction of higher alcohols content in the presence of sulfite, probably because sulfite causes the destruction of thiamine as shown by BORENSTEIN (1975):

TABLE 3 - Effect of micronutrient deficiency on yeast growth, ethanol and pyruvic acid production during alcoholic fermentation (Each result is the average of six determinations ± standard error).

Treatments	Yeast growth	Ethanol	Pyruvic acid
	mg/100 ml	% volume	mg/liter
Complete	342 ± 20	6.65 ± 0,08	96 ± 5
- Boron	344 ± 17	6.65 ± 0.08	93 ± 6
- Manganese	340 ± 25	6.63 ± 0.07	96 ± 10
- Zinc	278 ± 32	6.73 ± 0.09	96 ± 8
- Iron	331 ± 20	6.63 ± 0.07	91 ± 8

TABLE 4 - Effect of micronutrient deficiency on higher alcohol (mg/litre) production (Each result is the average of six determinations ± standard error).

Treatments	alcohols		
	n-propyl	isobutyl	isoamyl
Complete	17 ± 3	34 ± 7	206 ± 24
- Boron	15 ± 3	32 ± 4	178 ± 32
- Manganese	15 ± 2	37 ± 4	201 ± 19
- Zinc	17 ± 1	14 ± 2	80 ± 9
- Iron	15 ± 2	32 ± 6	176 ± 23

TABLE 2 shows a higher production of isobutyl and isoamyl alcohols in pyridoxal deficiency because this vitamin is essential for transaminase activity (DIXON & WEBB, 1964) and more ketoacids would be available for decarboxylation and reduction instead of for amino acids synthesis.

In summary, these data show that *S.cerevisiae* M-300-A requires the vitamins thiamine, pantothenic acid and pyridoxal from exogenous sources and that it does not need biotin for growth and the production of higher alcohols.

It was found that yeast growth was not inhibited when boron, manganese and iron were missing (TABLE 3). These data confirmed the observations with brewers yeast related by NORDSTROM (1964). When no zinc was added to the medium, growth was reduced by 18.7% and was followed by higher ethanol production. No effect of micronutrients was observed in relation to pyruvic acid formation (TABLE 3). Zinc is essential for several enzymes activities such as ethanolic dehydrogenase, aldolase, alcaline phosphatase (GOTTSCHALK, 1986), DNA and RNA polymerase (AULD et al., 1976), therefore explaining the less amount of biomass formed when zinc was deficient.

The lack of response to iron, boron, and manganese could be due to their presence as impurities in the chemicals used, to an accumulation in the yeast during propagation or even to a lesser need for these micronutrients.

TABLE 4 shows the effect of micronutrient deficiency on higher alcohol production. N-propyl alcohol levels were not

affected by any deficiency. Of all micronutrients studied only zinc was necessary for higher alcohol production. A significant reduction of isobutyl and isoamyl alcohols was found with zinc deficiency. The reduction of yeast growth and higher alcohol production coud be attributed to a lower absorption of sugar, as it has been shown by VISURI & KIRSOP (1970) or reduction of energetic metabolism (LAWFORD & PIK, 1980). Also the less amount of isobutyl and isoamyl alcohols may be partly due to a lower activity of ethanolic dehydrogenase which requires zinc as a cofactor (GOTTSCHALK, 1986).

ACKNOWLEDGEMENTS

This work was supported FERMENTEC-Assessoria em Fermentação Alcoólica (Brasil). The author also likes to thank Dr. Zilmar Ziller Marcos for reading and correcting this manuscript and Mrs. A.V.K.O. Annicchino and Mr. Luis Lucatti for technical assistance.

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Received in May 26, 1993
Accepted in July 30, 1993

Enviado para publicação em 26.05.93
Aceito para publicação em 30.07.93