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Full Length Research Paper

Optimization of biomass and dihydroorotase (DHOase) production by *Saccharomyces cerevisiae* MNJ3 (pMNJ1)

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Growth conditions which maintains DHOase overproduction by *Saccharomyces cerevisiae* MNJ3 (pMNJ1) and allow sufficient biomass production to ensure DHoase's purification were investigated. We used as basal medium the Yeast Carbon Base (YCB; Difco), especially designed for studies of nitrogen metabolism in yeasts. Thus the effects of an additional carbon source (glucose or citric acid) and the influence of the nitrogen source (asparagine or ammonium sulphate) as well as that of pH on growth of this strain were studied by supplementing the Yeast Carbon Base medium (Difco) with the above components. Cells were collected and DH0ase activity was determined. This study showed that the best conditions for biomass production required supplementation of YCB with asparagines 5 g/l, glucose 20 g/l at pH 5.5 (M12 medium). In this medium DHOase activity decrease with time; thus in terms of DHOase yield, the best time for havesting cells is reached after 30 to 38 h of growth.

Key words: Biomass production, dihydroorotase, optimization, *Saccharomyces cerevisiae.*

INTRODUCTION

The *de novo* biosynthesis of pyrimidine nucleotides is catalyzed by six enzymes encoded by the *pyr* genes. Dihydroorotase (DHOase) (EC 3.5.2.3) catalyzes the third reaction of the pathway, the cyclization of N-carbamyl-L-aspartate, to form L-5,6 dihyroorotate. In mammals, DHOase is part of a multienzymatic protein, ME *pyr*1-3 (Shoaf and Jones, 1971), which also contains a carbamyl phosphate synthetase (EC 2.7.2.9) and an aspartate transcarbamylase (EC 2.1.3.2) which catalyze the first two steps of the pathway. This protein, also called CAD or dihydroorotate synthetase, is organized into discrete structural domains, each having a distinct function (Mally et al., 1981; Grayson and Evans, 1983). In contrast, the bacterial DHOases are separate monofunctional proteins. First identified in *C. oroticum* (Liebermann and Kornberg, 1954), this enzyme specified by the *pyr*C gene has been isolated from this organism (Taylor et al., 1976) and from *E. coli* (Sander and Heeb, 1971; Lee et al., 2007). The amount of dihydroorotase (DHOase) in *E. coli* cells is generally low (Lee et al., 2007). For enzyme production from *E. coli* cells, Brown and Collins (1986) cloned the *E. coli pyr*C gene and overexpressed it in the *E. coli* RLM 569 *pyr*C strain. In Yeast the two first enzymatic activities are carried by a bifunctionnal protein, coded by the gene URA2. In *S. cerevisiae*, the URA4 gene encodes the monofunctionnal yeast DHOase (Lacroute, 1968). Chevallier and others (Chevallier et al., 1980) built a chimeric plasmid in order to clone yeast genes and to overproduce their corresponding protein. In 1988, Guyonvarch et al. (1988) published the sequence of the URA4 gene. They cloned the URA4 gene on a chimeric plasmid, and obtained a *S. cerevisiae* transformed strain which over-produced dihydroorotase when the strain was cultivated in synthetic medium. In this organism, the regulatory model of the pyrimidine pathway assumes that the first enzyme is subject to feedback inhibition and repression by the end product, e.g UTP (Lacroute, 1968). We pre-

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Table 1. Optimization of growth conditions by factorial design.

Medium code	Medium composition (g/l) and pH			Growth (absorbances after)	
	Nitrogen (g)	pH	Additional carbon (g)	16 h	38 h
M1	$(NH_4)_2SO_4$ (5)	6.2	None	0.39	5.00
M2	Asparagine (5)	6.2	None	0.49	8.89
M3	$(NH_4)_2SO_4$ (5)	5.5	None	0.350	8.32
M4	Asparagine (5)	5.5	None	0.44	5.55
M5	$(NH_4)_2SO_4$ (5)		Glucose (10)	0.56	7.25
M6	Asparagine (5)	6.2	Glucose (10)	0.528	10.22
M7	$(NH_4)_2SO_4$ (5)	5.5	Glucose (10)	0.440	9.98
M8	Asparagine (5)	5.5	Glucose (10)	0.56	7.55
M9	$(NH_4)_2SO_4$ (5)	6.2	Glucose (20)	0.430	10.90
M10	Asparagine (5)	6.2	Glucose (20)	0.56	7.80
M10	Asparagine (5)	6.2	Glucose (20)	0.53	8.20
M10	Asparagine (5)	6.2	Glucose (20)	0.51	8.20
M11	$(NH_4)_2SO_4$ (5)	5.5	Glucose (20)	0.40	7.95
M12	Asparagine (5)	5.5	Glucose (20)	0.66	12.07
M12	Asparagine (5)	5.5	Glucose (20)	0.60	11.93
M12	Asparagine (5)	5.5	Glucose (20)	0.60	12.14
M13	$(NH_4)_2SO_4$ (5)	6.2	Citric acid (5)	0.24	8.46
M14	Asparagine (5)	6.2	Citric acid (5)	0.22	4.50
M15	$(NH_4)_2SO_4$ (5)	5.5	Citric acid (5)	0.40	5.05
M16	Asparagine (5)	5.5	Citric acid (5)	0.50	10.49
M17	$(NH_4)_2SO_4$ (5)	6.2	Citric acid (10)	0.20	7.70
M18	Asparagine (5)	6.2	Citric acid (10)	0.32	4.40
M19	$(NH_4)_2SO_4$ (5)	5.5	Citric acid (10)	0.37	4.65
M20	Asparagine (5)	5.5	Citric acid (10)	0.54	10.39

sented here the selection of growth conditions which maintains DHOase overproduction by this clone and allow sufficient biomass production to ensure the purification of the DHOase.

MATERIALS AND METHODS

Materials and media

The two strains of *S. cerevisiae*, a wild type (FL100) and a mutant strain (a, ura4) transformed by the pFL1 plasmid containing the URA4 gene MNJ3 (pMNJ1), were kindly provided by F. Lacroute.

The Yeast Carbon Base (YCB; Difco) containing 10 g/l dextrose was used and supplemented with various components according to the objective of the culture:

- YCB1 medium: Yeast Carbon Base, supplemented with $(NH_4)_2SO_4$, (5 g/l); pH 6.2.
-YCB2 agar medium: YCB1 medium supplemented with yeast extract (0,0025%), and solidified with agar (2%); pH 6.2.
- M1 to M20 medium: designed to the effect of an additional carbon source and the influence of the nature of the nitrogen source as well as that of pH on growth of *S. cerevisiae* MNJ3 (pMNJ1)

Optimization of growth conditions

The effect of an additional carbon source and the influence of the nature of the nitrogen source as well as that of pH on growth of *S. cerevisiae* MNJ3 (pMNJ1) were studied using a factorial design (Box, 1978). We used as basal medium the Yeast Carbon Base (YCB; Difco), especially designed for studies of nitrogen metabolism in yeasts. After adjusting YCB medium pH to 5.5 or 6.2, we added a nitrogen source (asparagine or ammonium sulphate) and the additional carbon was either glucose or citric acid at concentrations indicated in Table 1. Since we did not study the effect of a combination of glucose and citric acid in a medium, there are 5 levels considering the carbon source. Thus, this factorial design requires 2 x 2 x 5 = 20 runs (media coded M1 to M20). Three replicates with M10 and M12 were planned for reproductibility.

Inoculum, grown for 18 to 19 h on YCB1 medium, was added so as to obtain 10^4 cells per ml in the prewarmed broths (300 ml), placed in shaker water bath (120 rpm) at 30°C. Absorbance (λ = 623 nm) was measured after 16 or 38 h. We have chosen to code pH 6.2, ammonium sulfate and citric acid by minus signs, and pH 5.5, asparagine and glucose by plus signs. For interpretation of results, we calculated the main effects of each factor or the interactions effects (Table 2) and built a geometric representation at a given pH according to indications of G.E.P. (Box, 1978).

Cells harvesting

Cultures inoculated as described above were grown in flasks of M12 medium placed in shaker water bath (120 rpm) at 30°C. Growth was followed from 14 h up to 52 h by measuring absorbance (λ = 623 nm). At selected times, cells were collected by centrifugation (5,000 g, 15 min at 4°C); the pellets were washed

Table 2. Effect of pH, nitrogen and carbon sources or their interaction effects on growth of *S. cerevisiae* MNJ3 (pMNJ1) at 30°C.

Factor or interaction	Mode of effect calculation	Factor or interaction effect on growth	
		After 16 h [a]	After 38 h [b]
Factor			
pH	(∑ D.OpH 5.5) /10 - (∑ D.OpH 6.2) /10	0.0710	0.6590
Nitrogen source	(∑ D.O(Asn) /10) - (∑ D.O(NH4) /10)	0.0970	0.6830
Glucose (10 g/l)	(∑ D.O(glucose 10 g/l) /4) - (∑ D.O(YCB seul) /4)	0.1050	1.8100
Glucose (20 g/l)	(∑ D.O(glucose 20 g/l) /4) - (∑ D.O(YCB seul) /4	0.0775	2.7975
Effect of increasing glucose level	(∑ D.O(glucose 20 g/l) /4) - (∑ D.O(glucose 10 g/l) /4)	-0.0275	0.9875
Citrate (5 g/l)	(∑ D.O(citrate 5 g/l) /4) - (∑ D.O(YCB seul) /4)	-0.0775	0.1850
Citrate (10 g/l)	(∑ D.O(citrate 10 g/l) /4) - (∑ D.O(YCB seul) /4)	-0.0600	-0.1550
Effect of increasing citrate level	(∑ D.O(citrate 10 g/l) /4) - (∑ D.O(citrate 5 g/l) /4)	0.0175	-0.3400
Interaction			
pH and nitrogen source	c	0.0430	1.3310
pH and glucose	d	-0.0375	0.2725
pH and citrate	e	0.0125	0.0900
Nitrogen source and glucose	f	0.0575	0.1775
Nitrogen source and citrate	g	0.0525	0.2400
pH, glucose and nitrogen source	h	-0.0075	3.0825
pH, citrate and nitrogen source	i	0.1200	-0.0900

a) SD = ± 0.03
b) SD = ± 0.15
c) (∑ D.OpH 5.5(Asn) /5 - ∑ D.OpH6.2(Asn) /5) - (∑ D.OpH 5.5(NH4) /5 - ∑ D.OpH6.2(NH4) /5)) /2
d) ((∑ D.OpH 5.5(glucose 20 g/l) /2 - ∑ D.OpH6.2(glucose 20 g/l) /2) - (∑ D.OpH 5.5(glucose 10 g/l) /2 - ∑ D.OpH6.2(glucose 10 g/l) /2)) /2
e) ((∑ D.OpH 5.5(citrate 10 g/l) /2 - ∑ D.OpH6.2(citrate 10 g/l) /2) - (∑ D.OpH 5.5(citrate 5 g/l) /2 - ∑ D.OpH6.2(citrate 5 g/l) /2)) /2
f) ((∑ D.O(Asn; glucose 20 g/l) /2 - ∑ D.O(NH4; glucose 20 g/l) /2) - (∑ D.O(Asn; glucose 10 g/l) /2 - ∑ D.O(NH4; glucose 10 g/l) /2)) /2
g) ((∑ D.O(Asn; citrate 10 g/l) /2 - ∑ D.O(NH4; citrate 10 g/l) /2) - (∑ D.O(Asn; citrate 5 g/l) /2 - ∑ D.O(NH4; citrate 5 g/l) /2)) /2
h) [(D.OpH5.5(Asn; glucose 20 g/l) - D.OpH6.2(Asn; glucose 20 g/l)) - (D.OpH 5.5(Asn; glucose 10 g/l) - D.OpH6.2(Asn; glucose 10 g/l))] /4 - [(D.OpH5.5(NH4; glucose 20 g/l) - D.OpH6.2(NH4; glucose 20 g/l)) - (D.OpH 5.5(NH4; glucose 10 g/l) - D.OpH6.2(NH4; glucose 10 g/l)] /4
i) [(D.OpH5.5(Asn; citrate 10 g/l) - D.OpH6.2(Asn; citrate 10 g/l)) - (D.OpH 5.5(Asn; citrate 5 g/l) - D.OpH6.2(Asn; citrate 5 g/l))] /4 - [(D.OpH5.5(NH4; citrate 10 g/l 20 g/l) - D.OpH6.2(NH4; citrate 10 g/l)) - (D.OpH 5.5(NH4; citrate 5 g/l) - D.OpH6.2(NH4; citrate 5 g/l)] /4

twice with 100 mM Tris,20 mM N-carbamyl-DL-aspartic acid buffer (pH 5.8) and kept at -20°C.

Dihydroorotase assay

The bioconversion of N-carbamyl-DL-aspartic acid to dihydro-L-orotate was measured using the pH 5.8 synthesis assay of Chevallier et al., (1980). The reaction mixture (1 ml) consisted of 10 µmol of 100 mM N-carbamyl-DL-aspartic acid (pH 5.8), 90 µmol of 100 mM KH_2PO_4 buffer (pH 5.8) and 5 µl of cell extract. Increase in absorbance (λ = 230 nm) was followed over a 3 - 4 min period (E_{230nm} (DHO)= 1.17 mM^{-1} cm^{-1}). A unit of DHOase is the amount of DHOase catalyzing the production of 1µmol of dihydro-L-orotate per min at 30°C. Specific activity is expressed as units of DHOase per mg of protein.

Protein concentrations were determined using the Bio-rad standard assay, and bovin serum albumin as standard (Sedmak and Grossberg, 1977).

RESULTS

Biomass production

The absorbances reported in Table 1 showing the highest values (biomass production) were noted with M12 medium. From Table 2, it can be noted that pH and the nature of nitrogen or carbon sources affected growth of *S. cerevisiae* MNJ3 (pMNJ1). But the main effects of these factors can not be interpreted individually (separately), and their influences must be considered jointly

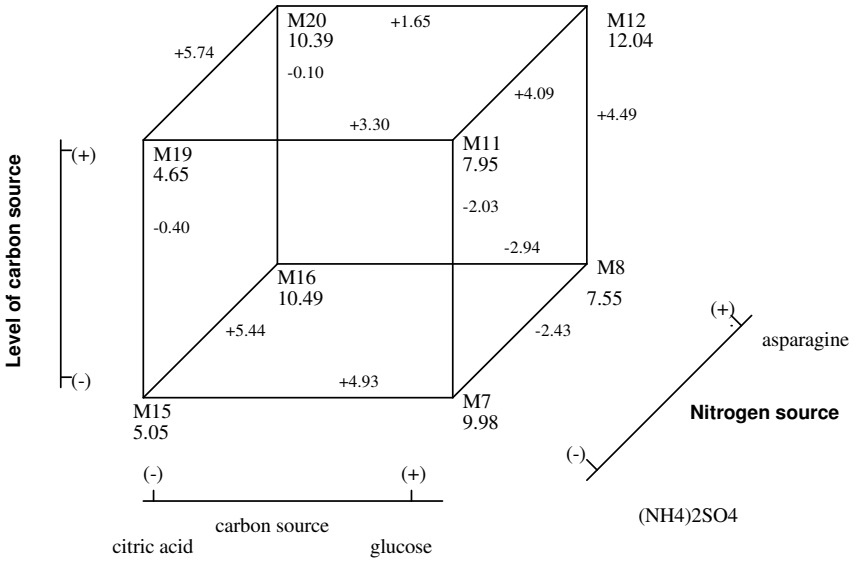

Figure 1. Geometric representation of main effects of nitrogen and carbon sources, level of carbon source and their interaction effects at pH 5.5.

Table 3. Kinetic production of dihydrororotase (DHOase) by *S. cerevisiae* MNJ3.

Age (h)	Wet weight (g/l)	Activity (U/g)	Protein (mg/g)	Sp. Act. (U/mg of protein)	Yield[a] (U/l)
22	2.6	21.65 ± 1.98	12.8	1.69	56
26	5.5	16.66±1.50	15.2	1.09	92
30	10.8	14.88 ± 1.72	17.4	0.85	161
34	13.5	13.75 ± 1.50	15.8	0.87	186
38	14.1	13.55 ± 0.61	14.3	0.95	191
46	16.3	9.79 ± 2.85	13.8	0.71	160
52	16.1	5.64 ± 0.59	7.6	0.74	91

[a]Yield is defined as the number of Dhoase units per liter of culture.

since there is interaction between them. From the geometric representation showing evidence for interaction effects (Figure 1) and from Table 2, we can state that:

- there is a positive interaction between pH and the nitrogen source, thus to say the combination pH 5.5 and asparagine 5 g/l favours (improves) growth of *S. cerevisiae* MNJ3 (pMNJ1) whatever the nature of the additional carbon source.
- Glucose is a better carbon source than is citric acid. Moreover, in contrast to citric acid where increasing its concentration from 5 to 10 g/l is not favourable for growth, increasing the glucose concentrations markly improves growth (compare M8 to M12).

In this study, the large positive interaction noted between pH, nitrogen source and glucose indicated that the best conditions for biomass production required a pH 5.5, asparagine 5 g/l and a high level of glucose as confirmed the high biomass production in M12 medium.

According to the kinetics of *S. cerevisiae* MNJ3 (pMNJ1) growth in M12 medium, a minimum of 22 h incubation is required for a limited biomass production. The production was markedly improved at 30 h and reached the maximum after 38 h incubation. The growth of *S. cerevisiae* wild type strain in M12 medium was followed and found similar to that of the transformed strain.

Dihydroorotase production

DHOase activities were determined using cells collected after 22, 26, 30, 34, 38, 46 and 52 h of growth. Table 3 shows that DHOase activity of the transformed strain decreased with time (varying from about 22 to 6 U/g after 22 and 52 h of growth respectively). In the same experimental conditions, the wild type strain produced a maximum of 1 U/g of cells (wet weight), for cells harvested after 22 h of growth.

In terms of DHOase yield, the increase from 56 to 191 U/l of culture reached after 38 h incubation was followed

by a regular decrease. To obtain the largest production of DHOase, in our experimental conditions the best time for harvesting the cells is between 30 to 38 h of growth.

DISCUSSION

The nutritional requirements of yeasts are complex since they need various vitamins and mineral salts. The Yeast Carbon Base fills these requirements but it lacks a nitrogen source. In this study, the best conditions for biomass production required a pH 5.5, asparagine 5 g/l and a high level of glucose. The fact that asparagine is directly used by yeasts whereas assimilation of ammonium sulphate requires glutamic acid, glutamine, can explain these results (Davis, 1986). The favourable role of asparagine results also from its utilization not only as nitrogen source but also as a secondary carbon source by decarboxylation and desamination. The enhancement of growth by high level of glucose can be explained by the fact that during exponential phase, yeast cells grow by fermentation whereas during diauxic phase and post-diauxic phase cells adapt to respiratory metabolism (Werner-Washburne et al., 1993).

Brown and Collins (1986) reported a 200-fold DHOase overproduction by *E. coli* RLM 569 *pyr*C, which allowed preparation of large quantities of DHOase. Here, we noted that the transformed strain produces 20-fold more DHOase than does wild type strain after 22 h of growth. This result, which agrees with those of Guyonvarch et al. (1988), shows that the URA4 gene is expressed in M12 medium as well as in the synthetic medium used by these authors. Event hough the degree of DHOase overproduction was less than that of *E. coli* RLM 569 *pyr*C, the cellular density obtained with *S. cerevisiae* MNJ3 (pMNJ1) strain was high enough to permit sufficient production of DHOase.

The amount of DHOase in cells showed a clear decrease in function of time. This is probably linked with processes involved in the yeast life cycle (metabolic behavior of *S. cerevisiae* cells). In fact, at slow growth rate (diauxic shift and post-diauxic phase), a proportion of the population differentiates into non-proliferating cells, stationary phase cells (Bugeja et al., 1982). Boucherie, (1985) and Fuge et al. (1994) showed that the diauxic shift is marked by a sudden alteration in the pattern of protein synthesis and repression of synthesis of most proteins. DHOase synthesis like other proteins synthesis might be repressed at slow growth rate. Since biomass production increases with time whereas DHOase yields decreases, a compromise has to be found in choosing the time for haversting the cells, and in our experimental conditions, it was between 30 and 38 h incubation.

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