0273-2289/97/6601-0031$9.75

The Effect of Formaldehyde on the Growth of *Candida diddensii 74-10* and *Candida tropicalis R-70*

CHRISTINA KATRANUSHKOVA,* BONKA TZVETKOVA, AND LILA LOSSEVA

Institute of Microbiology, Bulgarian Academy of Sciences, Acad. G. Bonchev str., bl.26, Sofia 1113, Bulgaria

Received December 30, 1994; Accepted January 25, 1996

ABSTRACT

The effects of formaldehyde on the growth of two strains of fodder yeasts *Candida diddensii 74-10* and *Candida tropicalis R-70* were investigated using the method of continuous cultivation under conditions of carbon limitation and at dilution rates of 0.1/h and 0.25/h. The results indicate that formaldehyde induces a decrease in the yield of biomass, but stimulates the synthesis of protein and RNA. The authors studied the activities of the following enzymes: NADPH-linked glutamate dehydrogenase, NADH-linked glutamate dehydrogenase, alanine dehydrogenase, glutamine synthetase, and glutamate synthase, which are utilized in nitrogen metabolism. The data obtained showed an increase in the activity of the glutamate dehydrogenase pathway of ammonium assimilation. It was also established that formaldehyde caused considerable changes in the micro-organisms at the higher dilution rate.

Index Entries: Formaldehyde; *Candida tropicalis; Candida diddensii*; nitrogen metabolism; protein; continuous cultivation.

INTRODUCTION

Acid wood hydrolisates or other wastes of plant origin can be used in some cases as culture media for the cultivation of fodder yeasts. In addition to reducing sugars, acid wood hydrolisates also contain formaldehyde, furfurol, organic acids, and other substances that cannot

*Author to whom all correspondence and reprint requests should be addressed. E-mail: microbas@bgearn.acad.bg

be completely separated. Thus, it is interesting to study their effects on the growth and protein synthesis of fodder yeasts. On the other hand, formaldehyde is a common product of the chemical industry that is often dispersed in ecosystems and because of its toxic and mutagenic properties, it represents a certain ecological hazard *(1)*. Therefore, formaldehyde degradation by bacteria and yeasts has been a subject of many recent studies *(2)*.

Formaldehyde can be assimilated by methylotrophic micro-organisms via the serine pathway by the ribulose monophosphate cycle or by the triokinase pathway as a sole source of energy.

It is known that when the formaldehyde content in the cell is increased it cannot be metabolized efficiently by inclusion in the ribulose monophosphate pathway, so it acts as an inhibitor. Formaldehyde is also an inhibiting agent when the activity of formaldehyde dehydrogenase is low and limits the consumption of formaldehyde as a substrate. This same behavior was found in nonmethylotrophic yeasts that did not use formaldehyde as a carbon source.

The object of the investigation were yeasts, which are very suitable models for investigation of the influence of formaldehyde on eucaryotic cells.

The aim of this study was to explain some aspects of the influence of formaldehyde on yeast growth, protein content, and the activity of some enzymes of nitrogen metabolism.

MATERIALS AND METHODS

Microorganisms and Cultivation

Two strains of fodder yeasts were used in our investigations, *Candida tropicalis, R-70*, with a fermentative type of metabolism (registered in National Bank for Industrial microorganisms and cell cultures, Sofia, Bulgaria, No NBIMCC 2083) and *Candida diddensii, 74-10*, with an oxidizing type of metabolism (registered in National Bank for Industrial microorganisms and cell cultures, Sofia, Bulgaria, No NBIMCC 2340).

In order to study the influence of formaldehyde on the different stages of yeasts' growth, the method of continuous cultivation was used. This method gives the opportunity to study the physiological responses of the micro-organisms to changes in the conditions of cultivation (i.e., addition of inhibitor) without changes in other parameters. The cultivation was achieved in chemostat with carbon limitation and at two dilution rates—of 0.1 h^{-1} and of 0.25 h^{-1}. The experiments were carried out in a fermentor (Ancum 2, Russian Academy of Sciences) with a working volume of 1.5 dm^3 at 38°C, pH 4.0, aeration 1 $dm^3/min.dm^3$ and agitation speed of 800 rpm.

Nutrient Medium

The control growth medium contained (g/dm^3) · glucose-13, $(NH_4)_2SO_4$-3.0, $MgSO_4 \cdot 7H_2O$-0.7, NaCl-0.5, $CaCl_2$-0.1, KH_2PO_4-1.0,

K_2HPO_4-0.1, biotine-0.04 mg/dm^3 and microelements (mg/dm^3)-$MnSO_4 \cdot 7H_2O$-0.4, $CuSO_4 \cdot 5H_2O$-0.4, $ZnSO_4 \cdot 7H_2O$-0.04, $FeSO_4$-0.2. For experiments 0.02% or 0.04% formaldehyde was introduced in the fermentor nutrient media.

Analytical Methods

After establishing a steady state, the yeasts were analyzed for the content of protein by Kjeldhal's method and the content of RNA by the method of Smith and Thannhauser *(3)* and Schneider *(4)*. The yield of biomass and the yield of protein were calculated from 100 g glucose. The protein synthesizing ability was calculated using the equation: A = protein content(%)/RNA(%) $\cdot$ $D(h^{-1})$. The rate of substrate assimilation was estimated using the equation: $Q = D\ (S_0 - S_1)/X$, where: S_0 and S_1 are the glucose content (g/dm^3) at the entrance and at the exit of the fermenter, respectively, X is the amount of cells (g/dm^3) and D is the dilution rate (h^{-1}). In order to investigate the impact of formaldehyde on the assimilation of nitrogen by yeasts, the amounts of the following enzymes were estimated: NADPH-dependent glutamate dehydrogenase EC 1.4.1.4 (NADPH-GDH), NADH-dependent glutamate dehydrogenase EC 1.4.1.2 (NADH-GDH), glutamate synthase EC 1.4.7.1 (GOGAT) and glutamine synthetase EC 6.3.1.2 (GS) using the method of Ferguson and Sims *(5)*, and alanine dehydrogenase EC 1.4.1.1 (ADH) by the method of Burk and Pateman *(6)*.

With the purpose of estimating these activities the cells were disrupted by a single passage in a mechanical disintegrator for 15 min. The obtained material was centrifuged at 14,000*g* for 30 min. The protein in the crude extract was determined by the method of Lowry *(7)*.

RESULTS AND DISCUSSION

When investigating the influence of a given inhibitor of growth, it is of great importance to choose the correct concentration, i.e., a concentration allowing the attainment of steady state. It was shown by Pilat and Prokop *(8)* that 0.01% formaldehyde is the minimum concentration limit above which formaldehyde acts as an inhibitor of yeasts' growth and 0.08% is the limit above which it acts in a bactericidal manner. Some of the preparatory investigations were carried out employing concentrations of formaldehyde from 0.005 to 0.08%. At 0.005%, significant changes in the growth were not observed, whereas at 0.08% it was impossible to reach a steady state. The concentrations used in the present work (0.02 and 0.04%) caused a decrease in the yield of biomass and increase of residual glucose in the culture media. This results allow us to consider the obtained cultures as inhibited ones.

When *Candida diddensii 74-10* were cultivated in medium with 0.04% formaldehyde at $D = 0.1\ h^{-1}$ the quantity of biomass decreased about 20%

Table 1
Effect of Formaldehyde on the Growth and Protein Content of *C. diddensii 74-10*

		D = 0.1 h^{-1}			D = 0.25 h^{-1}		
		Control	Formaldehyde		Control	Formaldehyde	
			0.02 %	0.04 %		0.02 %	0.04 %
1.	Biomass, g/dm^3	8.14	6.85	6.50	7.84	7.12	3.95
2.	Yield of biomass, %	61.0	58.0	56.0	63.5	63.0	56.0
3.	Rate of substrate assimilation, h^{-1}	1.3	1.5	1.8	3.8	3.9	4.2
4.	Protein, %	34.4	47.6	31.6	33.7	36.2	34.2
5.	Yield of protein, %	23.6	28.6	15.1	21.9	21.9	15.0
6.	RNA, %	3.70	4.80	1.98	3.34	6.29	1.97
7.	Protein - synthesizing ability, h^{-1}	1.14	0.99	1.59	1.02	1.43	1.73

and the yield of biomass decreased by 10% (Table 1). The amount of residual glucose in the culture medium increased to 1.5 g/dm^3. This is an indication that the yeast population is inhibited under these conditions, since the content and yield of biomass from 100 g of added glucose decreased. The decrease was a result of the impact of formaldehyde and was not because of the lack of substrate. These observations allow the conclusion that the cells were in a state of unspecific stress. Under these conditions, there was an increased substrate loss, which is necessary for the preservation of the cell's vital functions, and the rate of substrate assimilation increased by 11.3%. There are many investigations that prove that the energy spent on the reduction of some preservatives or inhibitors is not available for growth *(9,10)*, consequently tension emerges in the adaptational and reserve mechanisms of cells under conditions of stress.

Along with these common symptoms of the impact of unspecific inhibitors, each of them can cause specific changes. In the investigations at 0.02% formaldehyde, together with the common symptoms related to unspecific inhibitors an increase of RNA and protein content in the cell (i.e., activation of protein synthesis), was also observed. This is probably a specific change, caused by formaldehyde. The protein-synthesizing ability of *C. diddensii 74-10* also increased. For this reason, it is supposed that the increased protein content is caused by the larger number of ribosomes and their higher activity. In previous studies on the influence of formaldehyde on the ultrastructure of the same strains, that a piling up of ribosomes in the cell was shown *(11)*. These conclusions may be linked to the study of Pilat and Prokop *(8)* where 0.005% formaldehyde caused some stimulation of *C. boidinii 11Bh* growth. The higher concentration of formaldehyde (0.04%) caused a decrease of the protein and RNA content.

For the cultivation of *Candida tropicalis R-70* in the presence of formaldehyde, increases in the protein and RNA content were observed even at the higher concentration of formaldehyde (0.04%) (Table 2). This is probably because of the better ability of the strain *Candida tropicalis R-70,*

Table 2
Effect of Formaldehyde on the Growth and Protein Content of *C. tropicalis R-70*

		$D = 0.1\ h^{-1}$			$D = 0.25\ h^{-1}$		
		Control	Formaldehyde		Control	Formaldehyde	
			0.02 %	0.04 %		0.02 %	0.04 %
1.	Biomass, g/dm^3	7.95	7.5	6.50	7.14	4.40	3.40
2.	Yield of biomass, %	61.6	61.0	56.6	57.0	41.0	38.0
3.	Rate of substrate assimilation, h^{-1}	0.16	0.18	0.19	0.44	0.57	0.66
4.	Protein, %	36.0	38.0	41.0	26.0	30.0	37.9
5.	Yield of protein, %	23.0	22.0	20.0	14.3	14.0	15.0
6.	RNA, %	3.2	3.3	4.0	2.8	6.3	7.6
7.	Protein - synthesizing ability, h^{-1}	1.0	1.0	0.85	2.13	1.05	1.0

having a fermentative type of metabolism, to adapt to the inhibitor's action.

Comparing the results for the influence of formaldehyde at the two different dilution rates it was established that the changes in the investigated parameters are more considerable at the higher dilution rate 0.25 h^{-1}. Consequently, in both strains, different changes were observed depending on the dilution rates at the same formaldehyde concentration. Both strains' cells were more stable at the lower dilution rate. A similar conclusion was reached by Chanet et al. *(12)*, who showed that *S. cerevisae* cells are more stable in a stationary phase. Similar results were observed when treating cells with heat and alkylizing agents *(13)*. However, formaldehyde's influence has yet another explanation: according to some studies, formaldehyde interacts with free amino-groups, resulting in the formation of amino-methylol compounds, which afterwards can react with nucleotides *(14)*. Thus, the different effects of formaldehyde can be regarded as a result of the different pull of free amino acids in the cells. As the amino acid pull is larger in the exponential phase compared with the stationary phase, the changes are more considerable at the higher dilution rate, respectively at higher growth rate.

The results mentioned so far clearly demonstrate formaldehyde's ability to shift cells away from their normal physiological state. In order to retain their vital functions a series of unspecific compensatory and adaptational changes occur within the cells, one of which is probably the activation of protein synthesis. This is why the focus was on the investigation of formaldehyde's influence on the activity of some enzymes of nitrogen metabolism.

For *C. diddensii 74-10* the changes observed were typical for growth under conditions of carbon limitation when protein synthesis is activated (Table 3). At $D = 0.1\ h^{-1}$ and 0.04% formaldehyde, NADPH-GDH increased 1.8 times, whereas at $D = 0.25\ h^{-1}$ it increased 5.9 times. Under these conditions, the activity of ADH at both rates decreased five times. Hence, the glutamate dehydrogenase pathway of nitrogen assimilation is activated while

Table 3
Effect of Formaldehyde on the Enzyme Activities of *C. diddensii 74-10*

		D = 0.1 h^{-1}			D = 0.25 h^{-1}		
	Enzymes	Control	Formaldehyde		Control	Formaldehyde	
	n mole.$mg^{-1}min^{-1}$		0.02 %	0.04 %		0.02 %	0.04 %
1.	NADPH - GDH	421.00	765.00	765.00	497.00	642.00	2361.0
2.	NADH - GDH	87.90	28.00	28.20	35.50	44.80	35.0
3.	GS	0.41	0.84	0.37	0.10	0.50	0.30
4.	GOGAT	33.10	47.70	103.10	38.80	71.30	325.00
5.	ADH	35.40	9.17	6.17	19.70	16.50	4.90

NADPH-GDH-NADPH-linked Glutamate dehydrogenase, NADH-GDH-NADH-linked Glutamate Dehydrogenase, GS-Glutamine Synthetase, GOGAT-Glutamate Synthase, ADH-Alanine Dehydrogenase.

the alanine dehydrogenase activity is decreased. Although the activity of GS increased, it is less probable that the GS/GOGAT system was activated. The very low activities of GS in conditions of nitrogen excess (as was the case with the experimental setting at carbon limitation) may explain this.

For *C. tropicalis R-70*, an activation of the glutamate dehydrogenase pathway of nitrogen assimilation was observed also (Table 4). The activity of NADPH-GDH at $D = 0.25\ h^{-1}$ increased nine times. In both strains, regardless of the different type of metabolism, a considerable increase in the activity of NADPH-GDH was observed when the culture was exposed to the influence of formaldehyde. At $D = 0.1\ h^{-1}$, glutamate synthase activity was not observed, even at the lower formaldehyde concentration, whereas at $D = 0.25\ h^{-1}$ the activity of GOGAT decreased. These facts show that the GS/GOGAT system was not activated in this case either. Povereny et al. *(14)* report similar results and mention the ability of methanol-assimilating micro-organisms to assimilate ammonia via the glutamate dehydrogenase pathway and not via the glutamine syntethase and glutamate synthase pathways. It is supposed that formaldehyde causes activation of the glutamate dehydrogenase pathways of ammonia assimilation in nonmethanol-utilizing yeasts.

The changes observed in the activities of the enzymes, except for the NADH-GDH, are similar to the changes occurring in the cells when the growth rate is increased, for example, at a higher dilution rate or under better nutritional conditions NADH-GDH activities increased in response to the addition of formaldehyde to *C. tropicalis R-70* i.e., catabolic processes are activated *(15,16)*. This effect is not clearly seen with *C. diddensii 74-10*, an increase of NADH-GDH activity only at $D = 0.25\ h^{-1}$ and 0.02% formaldehyde was noticed.

Formaldehyde's impact on both strains causes similar changes in the activities of the investigated enzymes; the exception being GOGAT. In the strain having the fermentative type of metabolism-*C. tropicalis R-70*, the

Table 4
Effect of Formaldehyde on the Enzyme Activities of *C. tropicalis R-70*

		D = 0.1 h^{-1}			D = 0.25 h^{-1}		
	Enzymes	Control	Formaldehyde		Control	Formaldehyde	
	n mole.$mg^{-1}min^{-1}$		0.02 %	0.04 %		0.02 %	0.04 %
1.	NADPH - GDH	45.20	309.20	284.40	334.00	2856.0	917.00
2.	NADH - GDH	17.90	16.50	51.30	14.00	18.00	40.0
3.	GS	0.09	1.22	0.25	0.48	0.57	4.00
4.	GOGAT	20.10	n.d.*	n.d.*	69.00	30.00	25.00
5.	ADH	9.34	6.50	4.90	7.50	2.70	3.00

n.d.*—not detected

NADPH-GDH-NADPH-linked Glutamate dehydrogenase, NADH-GDH-NADH-linked Glutamate Dehydrogenase, GS-Glutamine Synthetase, GOGAT-Glutamate Synthase, ADH-Alanine Dehydrogenase.

activity of GOGAT is not present or shows a tendency to decrease, while in the strain having an oxidizing type of metabolism-*C. diddensii 74-10*, the activity of GOGAT increases.

To summarize, the presented results indicate that formaldehyde at the used concentrations (0.02% and 0.04%) causes a decrease of the biomass yield, changes of protein content in *C. diddensii 74-10* and *C. tropicalis R-70* and activates catabolic processes as well as the glutamate dehydrogenase pathway of ammonia assimilation.

REFERENCES

1. Grafstrom, R. C., Curren, R. D., Yang Li, L., and Harris, C. C. (1985), *Science* **228,** 89.
2. Adroer, N., Cases, C., de Mas, C., and Sola, C. (1990), *Appl. Microbiol. Biotechnol.* **33,** 217.
3. Schmidt, G. and Thannhauser, S. (1945), *J. Biochem.* **161,** 83.
4. Schneider, W. C. (1945), *J. Biochem.* **161,** 293.
5. Ferguson, A. R. and Sims, A. P. (1975), *J. Gen. Microbiol.* **80,** 159.
6. Burk, R. and Pateman, J. (1962), *Nature* **196,** 450.
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265.
8. Pilat, P. and Prokop, A. (1976), *Folia Microbiol.* **21,** 4, 306.
9. Warth, A. D. (1988), *Appl. Envi. Microbiol.* **54,** 2091.
10. Moon, N. J. (1983), *J. Appl. Bacteriol.* **55,** 453.
11. Mihailova, L., Losseva, L., Katranushkova, Ch., and Tzvetkova, B. (1987), *Acta. Microbiol. Bulg.* **20,** 25.
12. Chanet, R., Irard, C., and Moustacchi, E. (1975), *Mutation Res.* **33,** 179.
13. Schenberg-Frascino, A. and Moustacchi, E. (1972), *Molec. Gen. Genet.* **115,** 243.
14. Povereny, A. H., Yu, A., Shishin, A. S., and Sinzinis, B. J. (1975), *Mutation Res.* **27,** 123.
15. Peters, J. and Sypherd, P. S. (1979), *J. Bacteriol.* **137,** N3, 1134.
16. Khale, A., Srinivasan, M. C., and Deshpande, M. V. (1992), *J. Bacteriol.* **174,** 3723.