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Assimilation Spectrum of the Yeast *Candida utilis* 49 Used for Producing Fodder Yeast from Synthetic Ethanol

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ABSTRACT. Oxidizing and assimilating ability of the yeast *Candida utilis* 49 was tested with 21 different low-boiling organic compounds which come as components of raw synthetic ethanol. The highest yields of yeast dry weight were obtained with ethanol (72.0%), propanol (48.2%), ethyl acetate (43.4%) and acetic acid (34.2%). To a minor extent, the yeast was capable of utilizing also 2-propanol, butanol and 2-butanol; it oxidized most of the compounds tested.

The rapid development of utilization of non-sugar and synthetic substrates for the production of microbial single cell protein (SCP) in the last five years has been reflected in a considerable number of studies of methanol metabolism in yeast and bacterial strains newly isolated from naturally occurring sources (McLennan *et al.*, 1973; van Dijken and Harder, 1974; Mimura *et al.*, 1974; Oki and Kitai, 1974; Reuss *et al.*, 1974; Snedocor and Cooney, 1974; Blagodatskaya *et al.*, 1975; Sahm *et al.*, 1975; Tezuka *et al.*, 1975; Volfová, 1975; Wagner and Levitch, 1975).

Many studies deal also with the utilization of ethanol, and several large research and industrial establishments are pursuing the industrial production of SCP from synthetic ethanol using yeasts of various genera (Kharat'yan *et al.*, 1974, 1975; Guiraud *et al.*, 1974; Masuda, 1974; Masuda *et al.*, 1974; Amano *et al.*, 1975*a*,*b*).

Relatively little attention was given to the microbial utilization of higher alcohols (Jackson, 1973; Mates, 1974; Matsumoto and Sato, 1974; Murooka and Harada, 1974), lower fatty acids (Huňková, 1972; Kinsel and Leathen, 1973; Sustina *et al.*, 1973; Dijkhuizen and Harder, 1975; Wadzinski and Ribbons, 1975) and carbonyl compounds (McGucken and Woodside, 1973; Ibragimova and Sakharova, 1974; Thomas and Russel, 1975; Veselov *et al.*, 1974).

Synthetic ethanol produced by ethylene hydration can contain, according to the conditions of synthesis and product refining, various organic compounds belonging to lower alcohols, ethers, aldehydes, ketones, *etc.* Literary data on the effect of these impurities on the production of SCP are scarce (Uher, 1973).

The present work was devoted to the investigation of the oxidation and assimilation of low-boiling organic compounds found as impurities in raw synthetic ethanol. We endeavoured to determine the oxidation and assimilation spectrum of the strain *Candida utilis* 49 which is being contemplated as a suitable strain for industrial production of SCP from synthetic ethanol.

MATERIALS AND METHODS

Microorganism and preparation of inoculum suspension. The yeast *Candida utilis* 49, from the collection of our institute, was maintained on malt agar slopes and tranferred monthly. Inoculum suspension was prepared by twice repeated 24-h cultivation in 500-ml boiling flasks containing 100 ml sterilized (20 min at 111590 Pa) medium containing 4.8 g $(NH_4)_2SO_4$, 0.65 g KH_2PO_4, 0.25 g $MgSO_4 . 7 H_2O$, 0.01 g $ZnSO_4 . 7 H_2O$, and tap water up to 1000 ml. The pH of the medium was 5.0. For the first cultivation the flasks were inoculated with 48-h-old colonies grown on agar slopes in a test tube, the inoculum for the second cultivation was one tenth of the volume of cultivation fluid from the terminated first cultivation.

After the second cultivation the cells were washed twice, centrifuged, and resuspended in 0.02M phosphate buffer (for oxidation tests) or in physiological saline (for assimilation tests).

Cultivation procedure and composition of media. Oxidation tests were carried out in 50-ml test tubes closed by ground glass stoppers, containing 10 ml yeast suspension (10 mg yeast dry weight/ml) in 0.02M phosphate buffer (pH 6.1) and 10 ml 1% (w/v) aqueous solution of the tested compound. The compounds under study included: methanol, acetaldehyde, diethyl ether, hexane, ethanol, 2-propanol, propanol, acetone, methyl ethyl ketone, ethyl acetate, 2-methylpropane-2-ol, 2-butanol, 2-methylpropane-1-ol, butanol, butyraldehyde, crotyl alcohol, crotonaldehyde, allyl alcohol, acrolein, acetic acid, and crotonic acid. The test tubes were incubated for 20—24 h in a thermostat at 30 °C, their contents filtered and the filtrates were used for the determination of acidity, content of residual substrate, and the formation of volatile product (by gas chromatography).

Assimilation of the compounds tested was done in a medium containing 2.18 g urea, 0.65 g KH_2PO_4, 0.25 g $MgSO_4 . 7 H_2O$, 0.01 g $ZnSO_4 . 7 H_2O$ and tap water ad 1000 ml, with pH 5.5. The medium was supplemented with 1% (w/v) solution of the compound in question and inoculated with 2 ml yeast suspension (100 mg yeast dry weight/ml). The total volume of cultivation fluid in 500-ml flasks was 104 ml. The flasks were cultivated for 20—24 h at 30 °C on a rotary shaker (oxygen transfer 100 mmol O_2 l^{-1} h^{-1}) and the contents were analyzed for pH, biomass yield, formation of products and residual substrate.

Chemicals. Refined hydrogenated ethanol (East-Bohemian Distilleries, Chrudim) was purified of carbonyl substances by boiling with 2,4-dinitrophenylhydrazine and by distillation. Crotonaldehyde, acrolein, allyl alcohol and butyraldehyde (obtained from the Faculty of Organic Chemistry and Technology, Institute of Technology, Prague) were purified by distillation; crotonic acid was purified by crystallization. All other chemicals, mostly of reagent grade purity (Lachema, Brno), were distilled.

Analytical methods. Dry weight. 10 ml yeast suspension was filtered in an S_4 filter crucible, the cells were washed with distilled water and dried to constant weight at 105 °C.

Total nitrogen was determined by Kjeldahl micromethod.

Ammonia nitrogen was determined by distilling 10 ml filtered medium with the addition of MgO and titrating the distillate with sulphuric acid with an indicator according to Ma and Zuazaga.

The level of residual substrates and products was assayed in filtrates or their distillates by gas chromatography on a modified Chrom II apparatus with flame-ionization detection (Laboratorní přístroje, Prague). The conditions were: glass column (3.0 m . 3 mm) filled with Porapak Q (80—100 mesh), column temperature 182 °C, injector chamber temperature 220 °C. Carrier gas flow rate: 26 ml N_2/min; 35 ml H_2/min;

TABLE I. Oxidation of major impurities of synthetic ethanol and some other metabolically significant compounds by resting cells of *Candida utilis*.

Substrate	Gas chromatography (%)				Unidentified compounds	pH
	Residual substrate	Products				
		Acetaldehyde	Ethanol	Acetic acid		
Methanol	94.8	—	—	—	—	6.0
Acetaldehyde	72.8		0.04	0.08	x_1	4.1
Diethyl ether	67.8	—	—	—	—	5.8
Hexane	N	—	—	—	—	6.0
Ethanol	82.4	0.004		0.015	—	5.1
2-Propanol	97.8	—	—	—	x_2	6.0
Propanol	97.2	—	—	—	x_3	5.2
Acetone	95.6	—	—	—	—	6.0
Methyl ethyl ketone	99.2	—	—	—	—	6.0
Ethyl acetate	55.4	—	0.03	0.04	—	4.2
2-Methylpropan-2-ol	98.4	—	—	—	—	6.0
2-Butanol	99.2	—	—	—	x_4	6.0
2-Methylpropan-1-ol	99.6	—	—	—	—	6.0
Butanol	89.4	—	—	—	x_5	5.2
Butyraldehyde	83.6	—	—	—	x_6, x_7	4.3
Crotyl alcohol	95.6		0.004	0.002	x_8, x_9	5.3
Crotonaldehyde	63.6	0.01	traces	0.004	x_{10}	5.8
Allyl alcohol	96.2	—	0.002	traces	x_{11}	6.0
Crotonic acid	N	—	—	traces	x_{12}	6.0
Acrolein	90.4	—	—	—	x_{13}	6.1
Acetic acid	N	—	—		—	6.1

N, the content of substrate was not quantitatively determined.

600 ml air/min. 4-μl samples were fed with 5 μl Hamilton syringe. Qualitative determination of individual compounds was done by comparing their retention times with corresponding reference substance. Quantitative data were derived from calibration curves for ethanol (at different plotter sensitivities) after multiplying the calculated area by correction factors reflecting the detector sensitivity to individual compounds.

RESULTS

Oxidation of major impurities in raw synthetic ethanol and some other metabolically significant substances

The results of gas-chromatographic analysis show (Table I) that 20—24 h of incubation at 30 °C caused in all media a drop in substrate concentration due partially to the oxidation of the substances under study, partially to losses caused by vapourization of the sample and a possible adsorption of the compounds on yeast cell surface. The most marked decrease in substrate level was observed with crotonaldehyde. Substrate consumption due to the formation of products such as acetaldehyde, acetic acid or ethanol was found with ethanol, acetaldehyde, ethyl acetate (hydrolysis), crotonaldehyde and crotyl alcohol, and to a minute extent also with allyl alcohol and crotonic acid. Some of the peaks could not be identified due either to the lack of corresponding reference substance or to excessively long retention times. The decrease in pH values pointed also to substrate oxidation.

TABLE II. Utilization of major impurities of synthetic ethanol and some other metabolically significant compounds by the yeast *Candida utilis* 49

Substrate	Yeast biomass yield (%) *	Residual substrate (%)	Unidentified compounds	Medium pH	Ammonia nitrogen in medium (mg/100 ml)
Methanol	0	N	—	5.5	2.8
Acetaldehyde	0	0.024	—	3.1	0.1
Diethyl ether	0.3	traces	x_1	6.2	2.0
Hexane	0	N	—	5.5	1.0
Ethanol	72.0	0.0	—	7.2	9.9
2-Propanol	1.0	0.016	x_2	7.8	17.4
Propanol	48.2	0.007	—	6.5	11.4
Acetone	0	N	—	5.5	2.1
Methyl ethyl ketone	0.2	0.23	—	5.6	2.1
Ethyl acetate	43.4	traces	—	6.8	3.8
2-Methylpropan-2-ol	0	0.462	x_3	5.5	1.5
2-Butanol	2.6	0.149	x_4	7.8	12.7
2-Methylpropan-1-ol	1.1	0.24	x_5	3.8	1.5
Butanol	0.6	N	x_6, x_7, x_8	3.3	1.3
Butyraldehyde	0	N	x_9	3.4	1.1
Crotyl alcohol	0	N	x_{10}	4.9	1.2
Crotonaldehyde	0	0.261	—	4.5	0.9
Allyl alcohol	0	0.098	x_{11}	5.3	0.5
Crotonic acid	0	N	x_{12}	4.9	0.5
Acrolein	0	N	—	3.6	0.5
Acetic acid	34.2	traces	—	7.9	3.7

* Yield referred to the amount of substrate added.

Utilization of major impurities and some other metabolically significant substances

The results of assimilation experiments are summarized in Table II. Strain *Candida utilis* 49 failed to grow on 1% methanol, 2-methylpropane-2-ol, allyl alcohol, crotyl alcohol, acetaldehyde, hexane, acetone, butyraldehyde, crotonaldehyde, and crotonic acid. The highest yields of yeast biomass were obtained with 20—24 h of cultivation on ethanol (72.0%), propanol (48.2%), ethyl acetate (43.4%) and acetic acid (34.2%). Negligible utilization was found also with 2-butanol (2.6%), 2-methylpropane-1-ol (1.1%), 2-propanol (1.0%) and butanol (0.6%). Most of the substances tested probably attain in 1% solutions inhibitory concentrations and would be likely to be better utilized at lower concentrations. The table documents that under cultivation conditions employed ethanol, propanol, ethyl acetate and acetic acid were practically completely utilized by the yeast. It is noteworthy that diethylether, methyl ethyl ketone and acrolein exhibited no toxic effect on the yeast. Acetaldehyde was detected (in trace amounts) only with crotyl alcohol and acrolein, traces of ethanol were disclosed in the medium with acetaldehyde and crotyl alcohol, and acetic acid appeared only in acetaldehyde-containing medium. Apart from these products, a number of unidentified, predominantly high-molecular, substances were also detected. The low level of ammonia nitrogen in the medium and the low pH value found with many examined substances attest presumably to the substrate inhibition of urease as compared *e.g.* to the high activity of this enzyme in the medium containing 2-propanol or 2-butanol. In contrast to ethanol, most substrates can be assumed to be degraded oxidatively at the expense of biomass formation.

DISCUSSION

Oxidation and assimilation tests served to determine the ability of *Candida utilis* 49 to grow on some organic compounds occurring either as impurities in raw synthetic ethanol or as intermediary metabolites during the cultivation of yeast on ethanol. The yeast *Candida utilis* grew satisfactorily on a medium with ethanol, propanol, acetic acid and ethyl acetate. To a lesser extent, the yeast was capable also of utilizing higher alcohols such as 2-butanol, 2-methylpropane-1-ol, 2-propanol and, on a minute scale, also butanol. The yeast did not utilize (or oxidize) methanol and a number of other low-boiling organic compounds. A similar conclusion, *viz.* that bacterial and yeast strains growing on ethanol do not grow on methanol, was reached also by other authors. The utilization of higher alcohols decreased with increasing length of their carbon chain. Mates (1974) reported a relatively lower inhibitory effect of secondary alcohols as compared to their primary counterparts. Murooka and Harada (1974) observed likewise a competitive inhibition of ethanol utilization by normal alcohols but not by isoalcohols or tertiary alcohols. Hori *et al.* (1969) found that butanol concentrations as low as 0.1M inhibit the formation of ribonucleic acid and protein synthesis. However, our experiments showed that both *n*-butanol and 2-butanol (secondary butyl alcohol) are, to a certain extent, utilized by *Candida utilis* 49. In contrast, the strain is unable to utilize butyraldehyde and crotonaldehyde. The ability of yeast strains of genera *Candida* and *Trichosporon* to utilize 2-butanol was described and patented by Matsumoto and Sato (1974).

Oxidation tests proved the biochemical transformation of acetaldehyde, ethanol, ethyl acetate, crotyl alcohol, crotonaldehyde, allyl alcohol, and other substances. The decreased pH values of the medium attest also to the formation of substances with acid reaction, notably acids.

Some of the compounds tested were not oxidized but they may probably undergo a co-oxidation in a mixture with ethanol. It might be worth-while to determine their effect on biomass production during ethanol utilization. The oxidation and utilization of the compounds tested in our study are inadequately known. Harada and Hirabayashi (1968) found that the yeast *Hansenula miso* IEO 0146, growing on ethanol, glycerol, acetic acid and lactic acid, oxidizes various glycols to produce corresponding acids. The same authors (1969) established also that the utilization of ethanol by the yeasts *Hansenula miso*, *Candida albicans* and *Saccharomyces oviformis* yields 5-acetoxy-4-ketohexane acid. Oki *et al.* (1968) and Ogasawara *et al.* (1974) found that the growth of some bacterial and yeast strains in media containing lower alcohols affords products such as amino acids, in particular alkylhomoserines, L-glutamic acid, and others.

The determination of assimilation spectrum of production strain has a considerable importance. Ethanol, propanol, 2-methylpropan-1-ol and other organic compounds represent easily accessible products of the petrochemical industry which can be used for the production of microbial proteins. The ability to utilize or transform in some other way the compounds tested above has not yet been explored in the yeast *Candida utilis* 49, though the knowledge of the assimilation spectrum is undoubtedly of prime importance for the rational production of SCP. Further study will be concerned with the effect of these compounds on ethanol utilization during the production of yeast biomass.

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