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Formation of Ethyl Acetate in *Hansenula anomala*

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The effects of aeration, pH, and concentrations of ethanol, acetaldehyde, and acetic acid on the production of ethyl acetate by washed *Hansenula anomala* cells were studied. Yeast cultivated in strictly aerobic conditions produced ethyl acetate from ethanol only in the presence of added acetic acid. The amount of ethyl acetate produced was closely related to the concentration of undissociated acetic acid in the medium and greatest when this concentration was 7.5–11.5 mM. The ester formation was inhibited by cysteine. A connection between the ester formation and enzyme induction is discussed.

Previous studies [1–6] have indicated that the formation of ethyl acetate in *Hansenula* is a result of a combined effect of two different enzymes, *viz.* an acetyltransferase catalyzing the reaction between acetyl CoA and alcohol:

$$CH_3COSCoA + C_2H_5OH = CH_3COOC_2H_5 + CoASH \quad (1)$$

and an esterase responsible for the hydrolysis:

$$CH_3COOC_2H_5 + H_2O = CH_3COOH + C_2H_5OH \quad (2)$$

The evidence for reaction (1) is indirect and based partly on the fact that the amount of ethyl acetate produced generally greatly exceeds the equilibrium concentration of the esterase-catalyzed reaction (2).[2,7] The presence of the esterase is indicated by the very low apparent pH-optimum of the ethyl acetate production, which is subject to the effect of esterase inhibitors observed with some strains of *Hansenula*.[1,3,5,6] An esterase was recently isolated in partly purified form from an organism resembling *Hansenula anomala*.[6] The purpose of the present study was to check the optimal conditions of ester formation in suspensions of washed *Hansenula* cells as a preliminary stage for the preparation of active cell-free enzymes.

MATERIALS AND METHODS

The yeast strain *Hansenula anomala* (obtained from Kluyvers laboratory in 1949) was maintained on wort agar until used. The cultivation of the cell mass was performed in five-liter jar fermentors with continuous stirring. The composition of the growth

medium was that given by Davies *et al.*[8] except that glucose was replaced by sucrose. Two culture types were used: (1) an *aerobic* culture into which about 1 vol. air/vol. medium/min was blown through sintered glass and (2) a *semiaerobic* culture aerated by about 0.1 vol. air/vol. medium/min through a glass tube. The cultivation time was generally 48–52 h at 27±1°C. The yeast was separated by centrifugation, washed in distilled water and stored at +1°C.

The experiments with washed yeast were performed according to Peel[2] in 50 ml Erlenmeyer flasks which were shaken 140 cycles/min. The amount of yeast suspension was equivalent to about 80 mg dry weight. The pH was adjusted with 3 ml of McIlvaine buffer. Acetic acid in the substrate was added in the form of potassium acetate buffer. The total liquid volume was 10 ml and the incubation time 60 min. All the experiments with washed yeast cells were performed in an air-conditioned dark room at 27±1°C.

The amounts of ethyl acetate in supernatants of the centrifuged (10 min at 10 000 *g*) yeast suspensions were determined with a ferric hydroxamate method.[2] The colour intensity was measured using a Klett-Summerson colorimeter (filter S54). The colorimeter readings were corrected for phosphate. Ethyl acetate in samples containing cysteine or broken cells was isolated by distillation.[2] Acetaldehyde present in concentrations exceeding 1 mM gave a distinct colour with the hydroxamate reagent. The effect of acetaldehyde was estimated by measuring the change in colour intensity and comparing this with the change of colour intensities of standard solutions. Ethyl acetate was identified also by gas chromatography.

The broken cell suspensions were prepared in 0.25–0.5 M sucrose solution according to Edebo.[9]

RESULTS AND DISCUSSION

The results of the experiments with washed yeast cells agree in general with the observations of other authors.[1–6] Some new facts became, however, apparent in the effects of pH and acetic acid on the ester formation. To a certain extent the observed differences may be traced to different methods of yeast cultivation, but the hereditary differences between yeast strains are also worthy of consideration.

Experiments with different gas phases (Table 1) confirmed previous observations[2,4] and indicated that the formation of ethyl acetate by the studied yeast strain was an aerobic process. The amount of ethyl acetate produced was found to exceed the equilibrium value of the reversible hydrolysis reaction by a factor exceeding 10^2. The ester production was inhibited by cysteine to a degree comparable with the suppressive effect of cysteine on yeast respiration[10] (Table 2).

Table 1. Effects of mixtures of oxygen and nitrogen on the formation of ethyl acetate in suspensions of washed *Hansenula anomala* cells. Semiaerobic yeast; 0.2 M EtOH; 0.008 M acetate buffer; pH 4.6.

Oxygen, per cent (v/v)	Ethyl acetate, μmoles/h/mg yeast dry weight
0	0.019
1	0.052
5	0.078
20	0.162
100	0.192

Table 2. Effect of L(+)cysteine on the formation of ethyl acetate in suspensions of washed *Hansenula anomala* cells in aerobic conditions. Semiaerobic yeast; 0.2 M EtOH; 0.008 M acetate buffer; pH 4.6.

Cysteine, M	Ethyl acetate, μmoles/h/mg yeast dry weight
0	0.230
0.001	0.117
0.002	0.085
0.005	0.066
0.010	0.047
0.100	0.027

The results of the experiments in which different concentrations of ethanol, acetaldehyde, and acetic acid were present revealed significant differences between yeasts cultivated with different aeration rates. Yeast grown in strictly aerobic conditions produced ethyl acetate from ethanol only in the presence of added acetic acid. Also with semiaerobic yeast the ester formation was distinctly enhanced by acetic acid (Fig. 1). The maximum yield of ethyl acetate was closely related to the concentration of undissociated acetic acid at the prevailing pH (Table 3). The divergent value at pH 7.2 was obviously a consequence of the high total acetate concentration. Inorganic salts (NaCl, KCl, and $MgCl_2$) also inihibited the ester production when they were present in concentrations exceeding 0.1 M.

Yeast grown in semiaerobic conditions produced small amounts of ethyl acetate from ethyl alcohol alone with an optimum ester yield at pH 4.6 (Fig. 2). The optimal concentration of ethanol both alone and in the presence of acetic acid was 0.2 M. Acetaldehyde in concentrations of 1—10 mM at pH 4.6 slightly stimulated the ester formation by semiaerobic yeast, but in concentrations above 10 mM or in the presence of acetic acid, acetaldehyde inhibited the ester production (Fig. 3). No production or utilization of ethyl acetate was observed with broken cells or subcellular fractions either alone or in the presence of various cofactors.

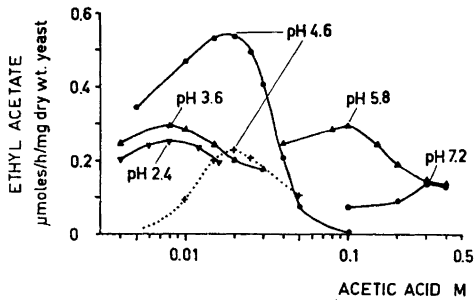

Fig. 1. Effect of acetic acid concentration and pH on the formation of ethyl acetate in suspensions of washed *Hansenula anomala* cells in aerobic conditions. EtOH 0.2 M; —— semiaerobic yeasts; ···· aerobic yeast.

Table 3. Effect of pH on the optimum concentration of acetic acid for the formation of ethyl acetate in suspensions of washed *Hansenula anomala* cells (Fig. 1).

Growth conditions of yeast	pH	Optimum concentration of acetic acid	
		Total concentration of acetic acid M	Concentration of undissociated acetic acid mM
Semiaerobic	2.4	0.008	8.0
	3.6	0.008	7.5
	4.6	0.015	8.6
	5.8	0.100	7.9
	7.2	0.300	1.0
Aerobic	4.6	0.020	11.5

As compared to some previous observations,[3,5,6] a different effect of pH and the inability of broken cells to utilize ethyl acetate suggested a difference in the utilization of ethyl acetate by the employed yeast strain. A closer study of the breakdown of ethyl acetate showed that it was influenced by the same factors as the ester formation. The breakdown of ester was oxygen-dependent and was inhibited by cysteine. It was also inhibited by ethanol, and the small concentrations of acetic acid which were optimal for the ester formation prevented utilization of the ester completely. Cultivation in aerobic conditions increased the ability of yeast to utilize ethyl acetate and by aerobic yeast the velocity of ester breakdown (0.51 μmoles/h/mg dry wt. yeast; pH 4.6; ethyl acetate initial concn. 0.01 M) approached the maximum velocity of ester formation. The effect of pH on the ester utilization resembled its effect on the ester formation (Fig. 2). On the whole the above data do not explicitly

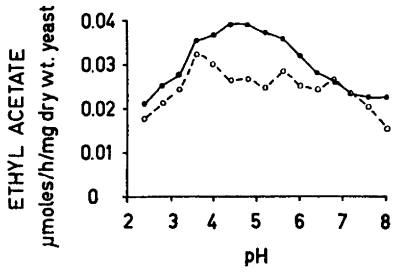

Fig. 2. Effect of pH on the formation and breakdown of ethyl acetate in a suspension of washed *Hansenula anomala* cells in aerobic conditions. Semiaerobic yeast; ● formation of ethyl acetate from 0.2 M EtOH; ○ disappearance of ethyl acetate, initial concentration 0.001 M.

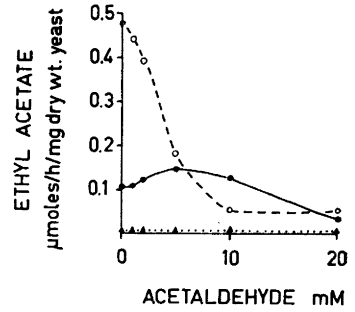

Fig. 3. Effect of acetaldehyde on the formation of ethyl acetate in suspensions of washed *Hansenula anomala* cells in aerobic conditions. EtOH 0.2 M; pH 4.6; ▲ aerobic yeast; ● semiaerobic yeast; ○ semiaerobic yeast in the presence of 0.02 M acetate buffer.

suggest the action of an esterase, which has been reported to be responsible for the breakdown of ethyl acetate in other strains of *Hansenula*. On the other hand, a direct reversal of a transacetylation mediated by CoA is unlikely for energetic reasons.[11] The reason for the discrepancy remains to be established.

The results indicate that the formation of ethyl acetate in *Hansenula* is closely connected with the metabolism of acetic acid in general and, in contrast to some previous views,[2] strongly suggest that acetyl CoA formed from extracellular acetic acid is used for the ester formation. As a consequence of aerobic adaptation, the activities of enzymes governing the oxidation of acetate and lipid synthesis are increased, while the activity of alcohol dehydrogenase is decreased.[12,13] These changes in enzyme activities obviously suppress intracellular acetyl CoA concentration and retard ethyl acetate production in aerobic yeast when ethanol is used as a substrate. A similar explanation is applicable in the case of acetaldehyde.

The close relationship between the ester formation and the dissociation of acetic acid seems to be comparable to some effects of acids on other organisms [14,15] and is obviously related to the permeability of cell membranes.[16]

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