

# IDENTIFICATION OF DEGRADATION PATHWAY OF VINYLACETATE USING BACTERIAL ISOLATE V2 AND CHARACTERIZATION OF THE INVOLVED ENZYMES

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## ABSTRACT

Vinyl acetate is a toxic substance, but has a high commercial value. In this study we show that vinyl acetate is subject to microbial degradation at rates of up to 6.38 and 1 mmol/h per g (dry weight) under aerobic and anaerobic conditions, respectively. It was hydrolyzed by bacterium V2 to ethanol, acetaldehyde and acetate. The enzymes involved in the metabolism of vinylacetate were vinyl acetate esterase, aldehyde dehydrogenase, and alcohol dehydrogenase, which localized in the cytoplasmic fraction. The Km values of vinyl acetate esterase and alcohol dehydrogenase were 6.13 mM and 0.24 mM, respectively. Vinyl acetate esterase hydrolyzed the ester to acetate and vinyl alcohol. The latter isomerized spontaneously to acetaldehyde and was then converted to acetate. The acetaldehyde was disproportionated into ethanol and acetate. The acetate was then converted to acetyl coenzyme A and oxidized through the tricarboxylic acid cycle and the glyoxylate bypass.

**Key words:** Vinylacetate, microbial degradation, vinylacetate-esterase, aldehyde dehydrogenase, alcohol dehydrogenase, bacterial isolate V2

## INTRODUCTION

Vinyl acetate (acetic acid ethenyl ester,  $\text{CH}_3\text{CO}_2\text{CH}=\text{CH}_2$ ) is a monocarboxylic, unsaturated aliphatic ester which will react violently with itself to yield polyvinyl acetate. Vinyl acetate is a clear, colorless liquid which can form flammable and explosive mixtures with air. The industrial use of vinyl acetate is for the production of polyvinyl acetate, polyvinyl alcohol and other polymers or copolymers. Its worldwide annual production capacity was about 3 million tons in 1982 and is still increasing (Roscher, 1983).

In a study examining the oxidation of several vinyl compounds by sewage under aerobic conditions, vinyl acetate was converted to  $\text{CO}_2$ , indicating its susceptibility to biological degradation (Beyer, 1984). The anaerobic

conversion of vinyl acetate to methane by municipal sludge has also been shown (Stuckey *et al.*, 1980,21). Fungi can grow in association with ethylene-vinyl acetate co-polymers (Griffin & Mivetchi, 1976), and polyvinyl acetate is degraded by strains of *Aspogillus* and *Penicillium* spp. (Trejo, 1988). Activity towards vinyl acetate has not been reported, however. In higher organisms, vinyl acetate was enzymatically hydrolyzed to acetaldehyde and acetate (Simon *et al.*, 1985), and no acetoxyoxirane (the epoxide of vinyl acetate) was formed (Simon *et al.*, 1986). Since we were not aware of studies dealing with the microbial metabolism of vinyl acetate in pure cultures, we have isolated active microorganisms towards vinyl acetate from environmental samples and characterized the metabolism of vinyl acetate by the bacterium V<sub>2</sub>.

## MATERIALS AND METHODS

**Media and growth conditions of Isolate V2.** The mineral medium of Schlegel *et al.* (Schlegel *et al.*, 1961) was used throughout, with the modification that phosphates were reduced to one third. The medium was supplied with 1 ml of trace element solution TS2 (Meyer and Schlegel, 1983) per liter reduced in sodium molybdate (0.03 g/liter). The pH was adjusted to 7.2. If not otherwise indicated, media were provided with 20 mM of the organic substrate. Media were solidified with 1.5% (wt/vol) agar (Difco). Vinyl acetate was supplied by evaporation from soaked filter paper attached to the inner side of the top dish. Petri dishes were kept upside down in taped plastic bags. Growth was at 30°C. Growth experiments under aerobic conditions were carried out in shaken 1-liter Erlenmeyer flasks containing 500 ml of mineral medium supplied with vinyl acetate, acetate, ethanol, or acetaldehyde as the sole sources of carbon and energy. Anaerobic growth was checked in 50-ml Erlenmeyer flasks containing 20 ml of the media indicated above. The flasks were kept shaken in desiccators and made anaerobic by using the GasPak anaerobic system (Merck). Growth was followed by A<sub>436</sub> measurements in a spectrophotometer (LKB).

**Degradation of vinyl acetate by bacterium V2.** Cells of bacterium V2 were incubated in serum-stoppered vials of 60 ml (total volume). Vinyl acetate (20 mM final concentration) was injected by using a syringe. Aerobic incubation was under air. Anaerobiosis was established by sparging the flasks with N<sub>2</sub> prior to the addition of vinyl acetate. Control assays were autoclaved for 20 min at 121°C prior to the addition of vinyl acetate. All flasks were kept shaken at 30°C. Samples (245 µl) were removed over time by means of a syringe and mixed with 5 µl of aqueous 2 M HCl to stop biological activities. Assays were subsequently clarified by low-speed centrifugation and analyzed with a gas chromatograph for the presence of vinyl acetate, acetate, acetaldehyde, and ethanol.

**Activities of resting bacteria towards vinyl acetate and its metabolic derivatives.** Resting cell suspensions of vinyl acetate-grown isolate V<sub>2</sub> were prepared as follows. Exponential cells

were washed by low-speed centrifugation in phosphate buffer and kept at -20°C until use. One gram of frozen cell paste was suspended in 0.3 ml of phosphate buffer before use. Of this suspension, 100 µl volumes were injected into serum-stoppered vials, incubated, and analyzed as described above for the degradation of vinyl acetate by bacterium V2. Acetaldehyde, ethanol, and acetate were also tested.

**Gas chromatographic analyses of metabolites.** Consumption or production of vinyl acetate, acetate, ethanol, and acetaldehyde were measured in a gas chromatograph (model 430; Packard) equipped with a flame ionization detector and a recorder (model 641; Packard). Samples (1 µl) were withdrawn from assays by use of a syringe and injected into the gas chromatograph operated under the following conditions: glass columns (0.25 in. [ca. 0.64 cm] by 3 m) filled with Porapak Q (80 to 100 mesh); carrier gas, N<sub>2</sub> at a flow rate of 11 ml/min; oven temperature, 200°C; injection port and detector temperature, 240°C. Under these conditions the detection limits were better than 1 µmol/ml.

**Preparation of bacterial extracts.** Suspensions of washed bacteria (ca. 40 g [wet weight]) in phosphate buffer, (about 40 ml) pH 7.5, were supplied with a few crystals of DNase I and passed four times through a precooled French pressure cell (American Instrument) operated at maximum pressure. The resulting crude bacterial extract was centrifuged at 11,400 x g for 40 min (rotor JA 20; Beckman) to remove unbroken cells and cell debris. Subsequently, the supernatant was subjected to ultracentrifugation at 203,400 x g for 3 h (Centricon T-1065, rotor TIT 45.94; Kontron) yielding a supernatant and a pellet, designated cytoplasm and cytoplasmic membranes, respectively. Membranes were suspended in phosphate buffer before use.

**Enzyme assays.** Enzyme activities were determined spectrophotometrically (model UV-120-02; Shimadzu) at 30°C.

**Vinylacetate esterase (EC 3.1.1.6).** Routinely, vinyl acetate esterase was assayed spectrophotometrically by measuring the formation of indoxyl from indoxyl acetate. To a cuvette containing 0.9 ml of phosphate buffer,



pH 7.2, 0.1 ml of an indoxyl acetate stock solution (100 mM in ethanol) was added. Reactions were started with extract as above. The increase of  $A_{375}$  was followed. The millimolar extinction coefficient of indoxyl was taken as 2.54/mmol per cm.

*Alcohol dehydrogenase* (alcohol:NAD(P)<sup>+</sup>, oxidoreductase, EC 1.1.1.71] and aldehyde dehydrogenase [aldehyde:NAD(P)<sup>+</sup>, oxidoreductase, EC 1.2.1.51]. Assays for alcohol dehydrogenase and aldehyde dehydrogenase contained 3 mM NAD<sup>+</sup> or NADP<sup>+</sup> and 1 mM alcohol or aldehyde in 1 ml of phosphate buffer, pH 7.5. Reactions were started by adding up to 10  $\mu$ l of extract. The formation of NAD(P)H + H<sup>+</sup> was followed at 365 nm. Acetyl coenzyme A (acetyl-CoA) synthetase (EC 6.2.1.1) was measured in a coupled assay with citrate synthase and malate dehydrogenase as described previously (Wiegant & de Bont, 1980). Isocitrate lyase (EC 4.1.3.1) was assayed by measuring the

formation of glyoxylic acid phenylhydrazone from glyoxlate (Dixon & Kornberg, 1959).

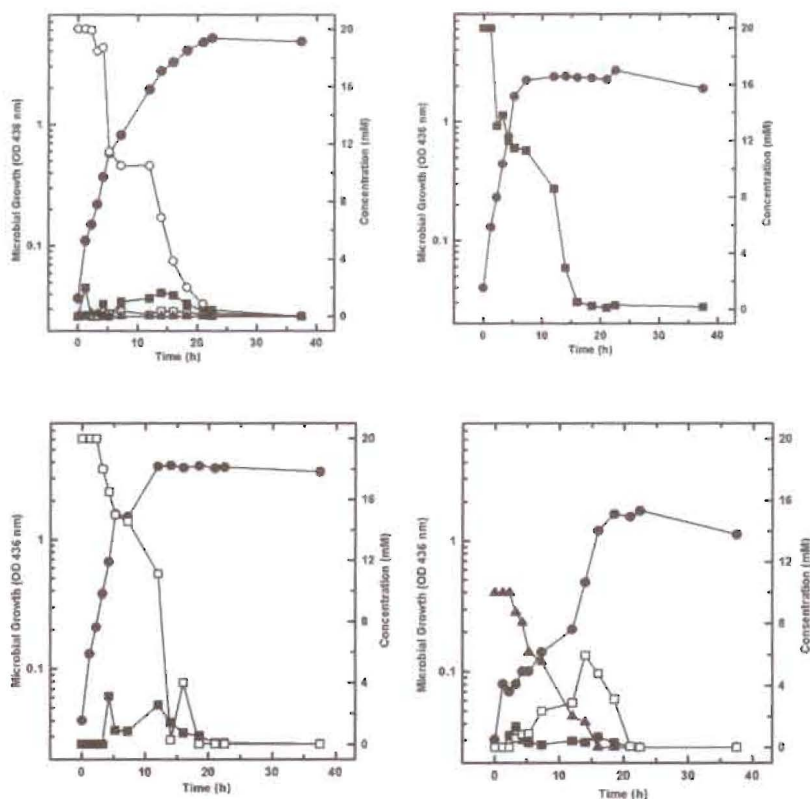
**Protein estimation.** The method of Bradford (Bradford, 1976) was employed for protein determination.

**Chemicals.** Vinyl acetate and indoxyl acetate were from Fluka, and acetaldehyde was from Merck. All other chemicals were purchased from commercial sources.

## RESULTS

### *Enrichment and isolation.*

Bacterium V2 was isolated from enrichment cultures inoculated with soil samples, as described in Materials and Methods. Bacterium V2 could grow on vinyl acetate as the sole source of carbon and energy. Besides vinyl acetate, growth was also supported by acetate, ethanol, acetaldehyde (Fig. 1).



**Figure 1.** Growth of bacterium V<sub>2</sub> on vinyl acetate and its metabolic derivatives. Growth was examined in 500-ml cultures supplied with vinyl acetate (A), acetate (B), ethanol (C), and acetaldehyde (D) as described in Materials and Methods. Symbols: (\*), growth at  $A_{436}$ ; (x), vinyl acetate; (Δ), acetate; (Δ), acetaldehyde; (□), ethanol.

### ***Growth on vinyl acetate.***

Isolate V<sub>2</sub> could grow aerobically in a medium containing vinyl acetate as the sole source of carbon and energy (Fig. 1A). Under these conditions, doubling times of 3 h and final optical densities of about 4 were obtained. Intermediates of vinyl acetate hydrolysis, such as acetate, ethanol, or acetaldehyde appeared in the culture broth only negligible amount.

### ***Growth on acetate, ethanol, and acetaldehyde.***

Growth of isolate V<sub>2</sub> with acetate (doubling time [t<sub>d</sub>] = 0.9 h) or ethanol (t<sub>d</sub> = 0.8 h) was much faster than with vinyl acetate (Fig. 1B). During growth with these substrates, no intermediate could be detected in the medium. Growth of isolate V<sub>2</sub> on acetaldehyde was biphasic (Fig 1D). The corresponding doubling times were 1.8 and 1.3 h, respectively. During the first growth phase, about 60 % of the acetaldehyde was utilized for growth and the remaining 40 % was converted to ethanol and excreted into the medium. The second growth phase was characterized by the utilization of ethanol, which obviously is a much better substrate than acetaldehyde (it is certainly less toxic). Only negligible amounts of acetate were formed under the conditions examined. Apparent millimolar growth yields (A<sub>436</sub>/mmol) of isolate V<sub>2</sub> were determined with vinyl acetate (0.143), acetaldehyde (0.08), ethanol (0.142), and acetate (0.0681).

### ***Intermediates of vinyl acetate metabolism.***

The metabolism of vinyl acetate was examined under conditions which did not allow complete oxidation but led to the excretion of

intermediates into the culture broth. For this purpose, resting cell suspensions of isolate V<sub>2</sub> were incubated with vinyl acetate or acetaldehyde under N<sub>2</sub>. At low concentrations (around 15 mM), vinyl acetate was stoichiometrically converted to ethanol and acetate (Table 1). At high concentrations (around 30 mM), in addition to ethanol and acetate, acetaldehyde also appeared. Similar results were obtained with resting cell suspensions in the presence of air (Table 1).

### ***Activities and other properties of enzymes involved in the metabolism of vinyl acetate.***

We were able to demonstrate in cytoplasmic fractions of isolate V<sub>2</sub> enzyme activities towards vinyl acetate and the derivatives of its metabolism suggested by the above experiments with growing and resting bacteria (Table 2). In addition to these, enzyme activities characteristic of the formation of acetyl-CoA from acetate and the glyoxylate bypass could also be demonstrated (Table 3).

Vinylacetate-esterase was constitutive enzyme that localized in the cytoplasmic fraction. Km and Vmax of the enzyme were 6.13 mM vinylacetate and 0,2 μmol vinylacetate/(min.mg), respectively. The K<sub>m</sub> values for vinyl acetate hydrolysis by microsomes, plasma (rat or human), purified acetylcholine esterase, butyrylcholine esterase, or carboxyl esterase ranged-from 0.65 to 77 mM (Simon, *et al.*, 1985). The affinity of vinyl acetate esterase from bacterium V<sub>2</sub> favourably compared with these figures.

Alcohol dehydrogenase catalysed the disproportionation of acetaldehyde to ethanol.

**Table 1.** Stoichiometry of products formed from vinyl acetate by resting cells of bacterium V<sub>2</sub>

Concn. of substrate (Vinyl acetate) (mM)	Concentration of product (mM)		
	Acetate	Ethanol	Acetaldehyde
18.0 (under N <sub>2</sub> )	33.3	5.6	0
14.5 (under air)	23.0	4.3	0
30.8 (under N <sub>2</sub> )	45.5	8.5	8.2
33.1 (under air)	37.5	9.4	10.8



The  $K_m$  value of alcohol dehydrogenase of Isolate V2 was very high (Table 2) compared with alcohol dehydrogenases from *Alcaligenes eutrophus* (5 mM) (Steinbuchel & Schlegel, 1984), *Acetobacter polyoxogenes* (1.2 mM) (Tayama, *et al.*, 1989). *Leuconostoc mesenteroides* (71 mM) (Bergmeyer, 1983). *Zymomonas mobilis* (1.7 and 100 mM) (Wills *et*

*al.*, 1981) and *Schwanniomyces castellii* (3.5 mM) (Mouillet-Loevenbruck *et al.*, 1989). As shown in Table 2, acetaldehyde dehydrogenase, which catalyzed the disproportionation of acetaldehyde to acetate, was also very active with acetaldehyde as a substrate, yielding the corresponding acids.

**Table 2.** Activities, specificities, and some kinetic properties of enzymes in cytoplasmic fractions of bacterium V<sub>2</sub>.

Enzyme or Substrate	Sp act. ( $\mu\text{mol}/\text{min}$ per mg of protein)	$K_m$ (mM)	$V_{\text{max}}$ ( $\mu\text{mol}/\text{min}$ per mg of protein)
Vinyl acetate esterase	0.10	6.13	0.19
Alcohol dehydrogenase	2.83	0.24	3.95
Aldehyde dehydrogenase	3.36	NL*	NL
Acetyl-CoA synthetase	0.05	ND**	ND
Isocitrate lyase	0.47	ND	ND

## DISCUSSION

### *Vinyl acetate is utilized by Isolate V2.*

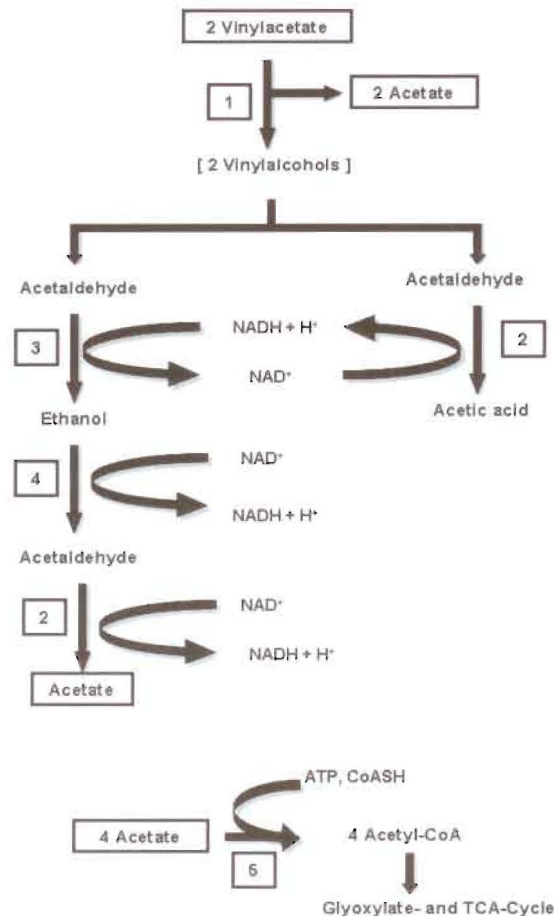
Isolate V2 is capable to utilize vinyl acetate as a sole source of carbon and energy for their growth and hydrolyse it without lag (Fig. 1). The reaction occurred under aerobic as well as under anaerobic conditions, although hydrolysis was considerably faster in the presence of air. Regardless of the conditions, the products were acetaldehyde, ethanol, and acetate.

### *Stoichiometry of vinyl acetate utilization.*

The pattern of products formed from vinyl acetate by resting cell suspensions of bacterium V<sub>2</sub> was determined by the concentration of vinyl acetate. At low concentrations, acetaldehyde was not formed, and the data of Table 1 support the following equation: 2 vinylacetate  $\rightarrow$  3.4 acetate + 0.6 ethanol. At elevated concentrations vinyl acetate was hydrolysed as follows (Table 1): 2 vinyl acetate  $\rightarrow$  2.6 acetate + 0.6 ethanol + 0.6 acetaldehyde. In growing bacteria, ethanol and acetaldehyde did not accumulate (Fig. 1) but were completely oxidized to acetate: vinyl acetate  $\rightarrow$  2 acetate.

### *Pathway for the metabolism of vinyl acetate.*

Nieder *et al.* (1990) propose a pathway for the conversion of vinyl acetate to acetate in bacterium V<sub>2</sub> (Fig. 2). Important consequences of this pathway are the complete conversion of 1 vinyl acetate to 2 acetates with the concomitant generation of one NAD(P)H + H<sup>+</sup>. The initial reaction of this pathway is the hydrolysis of one molecule of vinyl acetate to acetate and acetaldehyde. The aldehyde is disproportionated into acetate and ethanol. The latter is subsequently oxidized via acetaldehyde to acetate. It is interesting to note that methyl acetate is cleaved by aerobic methylotrophic bacteria into acetate and methanol (Rakov *et al.*, 1990). As with bacterium V<sub>2</sub>, the acetate is assimilated through the tricarboxylic acid cycle and glyoxylate bypass. Depending on the organism, either methanol dehydrogenase and the ribulose monophosphate cycle or alcohol oxidase and the serine pathway are employed for the assimilation of methanol (Rakov *et al.*, 1990). Esterase activities towards naphthyl and nitrophenyl esters of fatty acids have been described in the anaerobic bacterium *Butyrivibrio fibrisolvens* (Lanz, 1973). Esterase



**Figure 2. Pathway of vinyl acetate metabolism in bacterium  $V_2$ .** Enzymes involved are vinyl acetate esterase (1), aldehyde dehydrogenase (2), alcohol dehydrogenase (3, 4), and acetyl-CoA synthase (5). TCA, Tricarboxylic acid (Nieder et al., 1990).

activity was also observed in other ruminal bacteria, e.g., *Bacteroides ruminicola*, *Selenomonas ruminantium*, *Ruminobacter amylophilus*, and *Streptococcus bovis*. Acetyl xylan esterase is believed to play an important role in the overall digestion of forage by these bacteria.

#### *The glyoxylate bypass is employed.*

The acetate derived from vinyl acetate was converted to acetyl-CoA since acetyl-CoA synthetase was active in cells of isolate  $V_2$  growing on vinyl acetate (Table 2 and Fig. 2). Subsequently, acetyl-CoA was oxidized by the enzymes of the tricarboxylic acid cycle (Table 2). The presence of isocitrate lyase suggested that the glyoxylate bypass was in operation (Table 2).

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