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Characterizing NAD-Dependent Alcohol Dehydrogenase Enzymes of *Methylobacterium extorquens* and Strawberry (*Fragaria* × *ananassa* cv. Elsanta)

PANAGIOTIS KOUTSOMPOGERAS,[†] ADAMANTINI KYRIACOU,[‡] AND IOANNIS ZABETAKIS*,[†]

Laboratory of Food Chemistry, Department of Chemistry, University of Athens, GR-157 71 Athens, Greece, and Department of Dietetics and Nutritional Science, Harokopio University of Athens, GR-176 76 Athens, Greece

The methylotroph *Methylobacterium extorquens* (strain with CABI registration number IMI 369321), which has been isolated from strawberry (*Fragaria* × *ananassa* cv. Elsanta) callus cultures, was grown on a mixture of methanol (0.25% v/v) and 1,2-propanediol (0.75% v/v). The microbial biotransformation of 1,2-propanediol to 2-hydroxypropanal (lactaldehyde) was studied. The bacterial alcohol dehydrogenase (ADH) enzymatic activities were assessed, and the optimum pH for ADH activity was found to be pH 6.0. Enzyme assays were carried out for both the bacterial and the strawberry extracts to define the best substrate specificity. For *Methylobacterium extorquens*, the best substrates were found to be methanol (K_m = 0.78 mM) and 1,2-propanediol (K_m = 15.84 mM), whereas for strawberries, 1-propanol (K_m = 3.54 mM) and ethanol (K_m = 6.66 mM) were the best substrates. A wide variety of metals as well as EDTA were shown to decrease the enzymatic activity. Furthermore, SDS−PAGE experiments showed molecular weights of 45.0 and 24.6 kDa for the alcohol dehydrogenases of *Methylobacterium extorquens* and *Fragaria* × *ananassa*, respectively.

KEYWORDS: Alcohol dehydrogenase, *Methylobacterium extorquens*, flavor, symbiosis, strawberry

INTRODUCTION

Methylotrophy is defined as the ability to grow at the expense of reduced carbon compounds containing one or more carbon atoms but no carbon−carbon bonds (*1*). Enzymes for the primary oxidation of C1 substrates such as methanol dehydrogenase and methylamine dehydrogenase have been characterized, and distinct modes of C1 assimilation, such as the ribulose monophosphate cycle and the serine cycle, have been discovered (*3*). Additional methylotrophic pathways have been discovered, which solved the long-standing mystery of formaldehyde oxidation in many methylotrophs (*4*) and novel pathways for primary C1 oxidation, such as the pathways for degradation of chlorinated methanes and methanesulfonic acid (*5*). The availability of two unfinished genome sequences (*1*) for the important model organisms *Methylobacterium extorquens* and *Methylococcus capsulatus* transformed our understanding of methylotrophy. Formate dehydrogenase (FDH) activity has been detected in most methylotrophs (*6*), and a few FDHs have been purified and analyzed (*7*). *M. extorquens* AM1 (*3*) exhibits two pathways by which formaldehyde can be oxidized to formate, one linked to tetrahydromethanopterin (H4MPT) and another linked to tetrahydrofolate (*1, 4*).

In an enlightening review on pink pigmented facultative methylotrophs (PPFMs) (*8*), the close plant−microbe interactions were described, and particular emphasis was paid to the mutual benefits to both plant and microbial cells. Methylotrophic bacteria are involved in many interesting in vivo interactions between plants and bacteria. The species of PPFMs is very significant biologically because of its intimate relationship with the plant (*8*). Such a close relationship was observed between strawberry and *M. extorquens* in callus cultures where the bacteria oxidized 1,2-propanediol to lactaldehyde (*9*). Our work has also demonstrated that 1,2-propanediol occurs in strawberry (*10*) and strawberry callus cultures can bioconvert lactaldehyde to 2,5-dimethyl-4-hydroxy-2*H*-furan-3-one (DMHF) (*11*) and 1,2-propanediol to DMHF-glucoside (*10*). Given that DMHF is one of the most important components of strawberry flavor (*12*), we are particularly interested in clarifying the role of the bacteria in the bioconversion of the strawberry-derived 1,2-propanediol to lactaldehyde. We have therefore studied and report here this bioconversion in suspension cultures of this methylobacterium. Existing evidence (*9*) suggests a possible cooperation between the strawberries and the *Methylobacterium extorquens*, regarding their alcohol dehydrogenase system, which affects the dehydrogenation (oxidation) of certain alcohols. Hence, the purification of the alcohol dehydrogenases (ADHs) from the bacterium and the strawberries would prove extremely valuable for studying this plant−microbe interaction.

* Corresponding author: Dr. Ioannis Zabetakis, Stratigou Makriyanni 6, GR-143 42 Nea Philadelphia, Athens, Greece. E-mail: izabet@chem.uoa.gr.
[†] University of Athens.
[‡] Harokopio University of Athens.

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Such a plant, *M. extorquens* symbiosis, was also found in buds of scotch pine (*13*).

Methylotrophic bacteria can use a diversity of alcohols as energy sources as short primary alcohols (e.g., methanol and ethanol) or various diols such as 1,2-propanediol and polyols (e.g., glycerol) (*14*−*19*). The first step in 1,2-propanediol oxidation involves either a dehydration or a dehydrogenation, yielding propanal or lactaldehyde, respectively (*18*). The majority of data on ADHs concern single-hydroxyl alcohols. Certain experiments performed on extracts of ethanol-grown *Desulfovibrio* strains have shown high NAD-dependent ethanol dehydrogenase activities (*16*).

It is also known that a large group of metal ions, especially heavy metals such as lead, deactivate ADHs, mostly by reacting with −SH residues of the enzymatic amino acid structure. These ions can also interact with free carboxyl groups of the enzyme, thus altering the enzyme's conformation, which will also lead to the enzyme's partial deactivation. Another important category of enzyme effectors is chelates (e.g., EDTA), which also affect the activation of the enzymes by blocking a metal (usually zinc) that occupies a central place within the enzyme's active site (*20*).

The aim of this study was to fully characterize and compare the bacterial and plant NAD-dependent alcohol dehydrogenases in order to comprehend whether and to what extent the oxidation of the flavor-related alcohols to the corresponding aldehydes is carried out by the bacterial enzymes or the plant ones.

MATERIALS AND METHODS

Chemicals. All chemicals used were of the highest purity commercially available from Chemicals Merck (Darmstadt, Germany), Sigma Aldrich (Saint Louis, MO) and Fluka (Buchs, Switzerland).

Organisms, Cultivation, and Cell Extract Preparations. The strain used was *M. extorquens* [laboratory collection, strain IMI 369321 (CABI registration numbers)], isolated from strawberry callus (*9*). *M. extorquens* was cultivated in a nutrient medium containing 0.75% (v/v) 1,2-propanediol, 0.25% (v/v) methanol, and 1.0% (w/v) peptone, as described previously (*11*). A calibration curve was plotted by measuring the absorbance at 550 nm (in a UV spectrophotometer from Bausch and Lomb) of individual dilutions of the cell suspension against the blank (i.e., without bacterial inoculum). The equation $Y = 7.29 \times 10^8 X + 144659$, where X represents the absorbance and Y represents the number of cells, was obtained with $R^2 = 0.9920$. The cell counts of *M. extorquens* cultures were calculated using the presented equation after absorbance measurements. Cells were harvested at the end of their exponential phase and then centrifuged (using a Gallenkamp centrifuge) at 7000 rpm for 10 min, washed twice with 0.1 M phosphate buffer (pH 8.0), recentrifuged, and stored in 50 mL of the same buffer. Small amounts of the bacterial pellet (2 g in 50 mL of 0.2 M phosphate buffer, pH 8) were prepared by ultrasonic disintegration (using a Branson ultrasound device) of cells under ice-cooling. The cell extract that was used for enzyme assay was stored at 2 °C. The enzyme solution from strawberries was produced by placing 200 g of strawberries (*Fragaria* × *ananassa*) into an OMNI Mixer Speed 2 program for 10 min. The pulp was then filtered under vacuum (with 401 Whatman filter paper), and the filtrate was stored at 2 °C.

Enzyme Assays. The enzyme assays are based on measuring the absorption at 340 nm for NADH (*20*). First, the reaction was started with the substrate instead of the enzyme. All enzyme experiments were conducted in three independent replicates. Values presented here are the mean values ± standard deviation for 95% confidence levels. Three different and independent bacterial cultures or fruit batches were assessed for each experiment. Each fruit batch was formed by pooling six different fruits. The standard dehydrogenation assays contained 35 mM phosphate buffer or 35 mM tris(hydroxyamino)methane (Tris) depending on the pH profile, 1 mM NAD, and 0.1 mL of enzyme extract (either from the bacterium or from the strawberry), in a total volume of 3 mL. The reaction was started by injecting the respective alcohol each time. The exact substrate concentration was methanol, 30 mM; ethanol, 25 mM; 1-propanol, 25 mM; 2-propanol, 25 mM; glycerol, 20 mM; 1,2-propanediol, 25 mM; 1-butanol, 5 mM; benzyl alcohol, 0.5 mM; formaldehyde, 20 mM. The influence of metal chelators (EDTA) and metal ions was tested by preincubation of the enzymatic extract with the compound for 5 min. In the case of metal ions especially, instead of phosphate buffer, an acetate buffer 35 mM was used. Moreover, the bacterial-cell-free extract was diluted with acetate buffer (0.5 M, pH 6) instead of phosphate buffer. Protein was measured using bovine serum albumin as a standard (*21*). More specifically, proteins were stained with Coomassie Brilliant Blue R-250.

pH Profile. Investigation of the pH profile of the enzyme was carried out by producing a series of buffer solutions, ranging from pH 2.0 to 12.0 with an increasing step of 2.0 pH units. For pH values ranging from 2.0 to 8.0, a 0.2 M phosphate buffer was used, whereas for pH values ranging from 10.0 to 12.0, a 0.2 M Tris hydrochloride was applied. The pH dehydrogenation assay was performed with methanol (100 mM) as the substrate. The pH was measured with an ORION 410 A pH meter.

Chromatographic Separation/Purification of ADHs. The chromatographic separation/purification of ADHs was performed on a SEPHADEX C-50 (Pharmacia, Uppsala, Sweden) column (*20*). Then, 3 g of a sonicated bacterial pellet (from *M. extorquens*) was dissolved in 4 mL of phosphate buffer (0.1 M, pH 2.0). Subsequently, the 4-mL portion was placed into the column, and fractions of approximately 2 mL were collected. A pH gradient (from pH 2.0 to pH 12.0 with an increasing step of 2.0 pH units) was then applied for a total volume of approximately 35 mL. Protein measurements were conducted by measuring the optical density at 280 nm. Additionally, ADH activities were measured (as the enzyme assay experiments were conducted). The most active fraction for ADH activity was then returned to the column. The same procedure as described above was followed again, and the most active fraction was once again collected. The procedure that was followed for the strawberry was exactly the same with the only difference being that, instead of taking 3 g of bacterial pellet and suspending it in 4 mL of buffer solution, the following steps were done: 100 g of strawberries was crushed in a mixer (OMNI MIXER) and filtered, and 5 mL of the filtrate was placed into the chromatographic column.

SDS−PAGE. Active fractions that were collected from the chromatographic separation (for both the bacterium and the strawberry) were subsequently subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) (*20*) in order to obtain their molecular weights (Sigma). To perform these experiments, the following preparations were made: 12.5% acrylamide gels were prepared according to Laemmli (*22*). Proteins were stained with Coomassie Brilliant Blue R-250. Protein markers (high and low range) were used as standards to establish the molecular weights of the unknown proteins. The device used for the electrophoresis was a mini-Protean from Bio-Rad. Values in parentheses indicate the molecular weights of the standard proteins used in this experiment (in kDa): myosin (205), β-galactosidase from *E. coli* (116), phosphorylase b from rabbit muscle (97), fructose-6-phosphate kinase from rabbit muscle (84), albumin bovine serum (66), glutamic dehydrogenase from bovine liver (55), ovalbumin from chicken egg (45), glyceraldehyde-3-phosphate dehydrogenase (36), carbonic anhydrase from bovine erythrocytes (29), trypsinogen bovine pancreas (24), trypsin inhibitor from soybean (20), α-lactalbumin from bovine milk (14.2), and aprotinin bovine lung (6.5).

Native PAGE. The same procedure as was carried out for SDS−PAGE was applied for native PAGE with the exception that no SDS was added in the solutions. The protein markers that were used here were produced as follows: bovine serum albumin (300 mg) and ADH from *Saccharomyces cerevisiae* (50 mg) were added to 100 mL of deionized water. Molecular weight values in parentheses in **Figure 5** below indicate the molecular weights of the standard proteins used in this experiment (in kDa): albumin bovine serum (66), ADH from *Saccharomyces cerevisiae* (43.2).

Extraction of Proteins from Native Electrophoresis Gel. The gel was initially cut with a razor into horizontal lanes. Each lane was subsequently placed into a buffer solution (50 mM Tris HCl, pH 9)

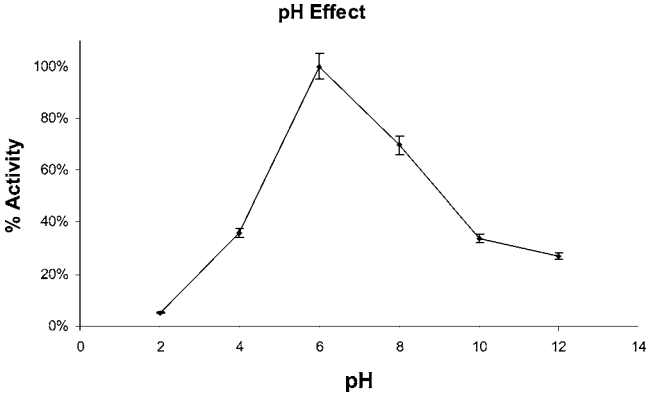

Figure 1. Effect of pH on alcohol dehydrogenase activity for *Methylobacterium extorquens*. The activity at pH 6.0 was considered as 100%. The error bars correspond to replicate measurements.

for 24 h at 0−4 °C. The extracted proteins (from both the bacterium and the strawberry) were then tested for ADH activity in the way we previously described.

HPLC Analysis of Lactaldehyde. The HPLC analysis of lactaldehyde was performed as described before (*23*). In detail, quantitative HPLC determinations were conducted using a Waters model 600E pump and a Waters model 996 photodiode array detector. A Techopak 10-ODS column (250 × 3.9 mm, HPLC Technology Ltd., Macclesfield, Cheshire, U.K.) was coupled to a M-bondapak guard column (30 × 4.6 mm, HPLC Technology Ltd., U.K.). The injection volume was 20 μL. The mobile phase was 60% H_2O/40% CH_3CN (isocratic, flow rate of 1.5 mL·min^{-1}, detection at 365 nm).

Quantitative Analysis of Lactaldehyde. Lactaldehyde was synthesized following the method of Zabetakis et al. (*11*). The crude bacterial enzyme lysate was incubated with 1,2-propanediol for 5 h, and at the end of the incubation period, dinitrophenylhydrazine (DNP) was added. After extraction with ethyl acetate and evaporation of the organic phase, the precipitate was subsequently dissolved in acetonitrile. It was finally analyzed by HPLC−PDA as described before (*9*). Linear responses from HPLC−UV analysis ($r = 0.9997$) were obtained for lactaldehyde in the concentration range 0.5−50 μg mL^{-1} in acetonitrile, and a standard curve was constructed. In all runs, the aldehyde was analyzed in the supernatant of the cultures. All analyses were carried out in triplicate, and the results are shown as average values ± standard deviation (95% confidence levels).

RESULTS

pH Profile, Physical and Catalytic Properties, Enzyme Assays. The pH profile of the ADH enzyme assay is shown in **Figure 1**. From these data, we concluded that the pH optimum is pH 6.0 [with methanol (100 mM) as the substrate], and all subsequent measurements were performed at this pH. NAD (as the sodium salt) was used as an electron acceptor, and the produced NADH was measured spectrophotometrically at 340 nm (*20*). One unit is the amount of enzyme that forms 1 μmol of NADH per minute.

The product of the oxidation of 1,2-propanediol was confirmed to be lactaldehyde using the methodology described before (*9*).

Apparent K_m values for *M. extorquens* and strawberries (*Fragaria* × *ananassa*), are presented in Table 1. The presence of Na^+, Cr^{3+}, Zn^{2+}, K^+, Zr^{2+}, Fe^{2+}, Ca^{2+}, Li^+, Pb^{2+}, and Ba^{2+} (all at 5 mM) resulted in the following relative activities for *M. extorquens* (control without addition of metal was considered as 100%): Na^+, 99%; Cr^{3+}, 16%; Zn^{2+}, 71%; K^+, 99%; Zr^{2+}, 22%; Fe^{2+}, 52%; Ca^{2+}, 98%; Li^+, 72%; Pb^{2+}, 2%; Ba^{2+}, 6%. For strawberries, the respective values were: Na^+, 99%; Cr^{3+}, 12%; Zn^{2+}, 83%; K^+, 98%; Zr^{2+}, 28%; Fe^{2+}, 39%; Ca^{2+}, 94%; Li^+, 68%; Pb^{2+}, 4%; Ba^{2+}, 5% (control without addition of metal was considered as 100%). The results are shown in the form of a graph in Figure 2A. Addition of metal chelators resulted in the following activities for *M. extorquens* (control without addition of EDTA was considered as 100%): EDTA (10.5 mM), 69%; EDTA (21 mM), 8%. For strawberries, the respective values were (control without addition of EDTA was considered as 100%): EDTA (10.5 mM), 75%; EDTA (21 mM), 56%. The results are shown in **Figure 2B**.

Table 1. Apparent K_m Values (mM) of Alcohol Dehydrogenase of *Methylobacterium extorquens* and Strawberry (*Fragaria* × *ananassa*)

substrate	***M. extorquens***	***F.* × *ananassa***
methanol	0.78 ± 0.00	9.69 ± 0.05
1,2-propanediol	1.65 ± 0.01	15.84 ± 0.02
glycerol	2.58 ± 0.02	18.60 ± 0.07
1-propanol	3.09 ± 0.02	3.54 ± 0.01
2-propanol	3.42 ± 0.03	11.46 ± 0.05
formaldehyde	4.46 ± 0.03	not detected
ethanol	5.38 ± 0.02	6.66 ± 0.03
benzyl alcohol	7.72 ± 0.09	19.96 ± 0.14
1-butanol	25.78 ± 0.23	19.93 ± 0.19

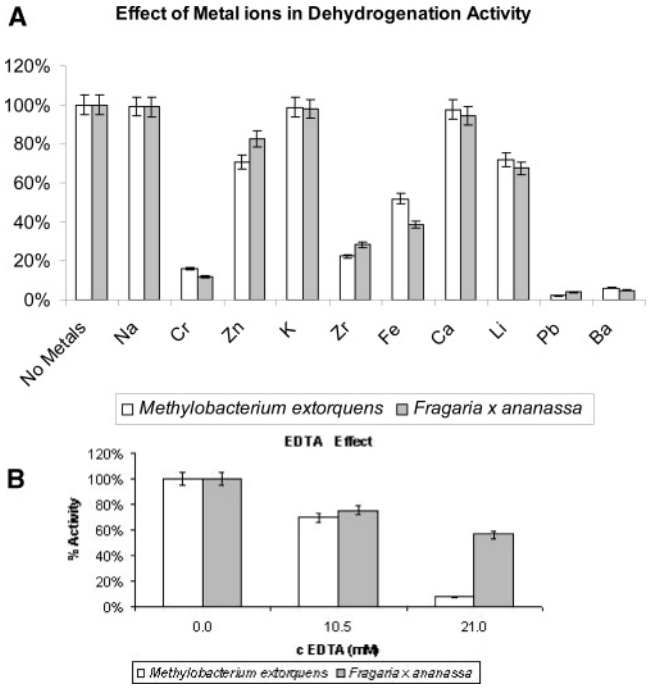

Figure 2. (A) Relative activities for the effect of metals on alcohol dehydrogenase activity. Control without addition of metal was considered as 100%. The error bars correspond to replicate measurements. (B) Relative percent effect of EDTA on alcohol dehydrogenase activity. Control without addition of EDTA was considered as 100%. The error bars correspond to replicate measurements.

Chromatographic Separation/Purification of ADHs. The protein contents of the crude extracts were 268.2 mg of protein for the bacterium and 185.5 mg of protein for the strawberry. For the bacterium, 20 fractions were collected during the first purification step, and the fraction with the highest dehydrogenation activity was returned to the column (2 mL) one more time. Fifteen more fractions were produced by this method. The fraction with the highest dehydrogenation activity from the second run was used for further SDS−PAGE. For the strawberry, 18 fractions were collected during the first purification step, and the fraction with the highest dehydrogenation activity was returned to the column (2 mL) one more time. Seventeen more fractions were produced by this method.

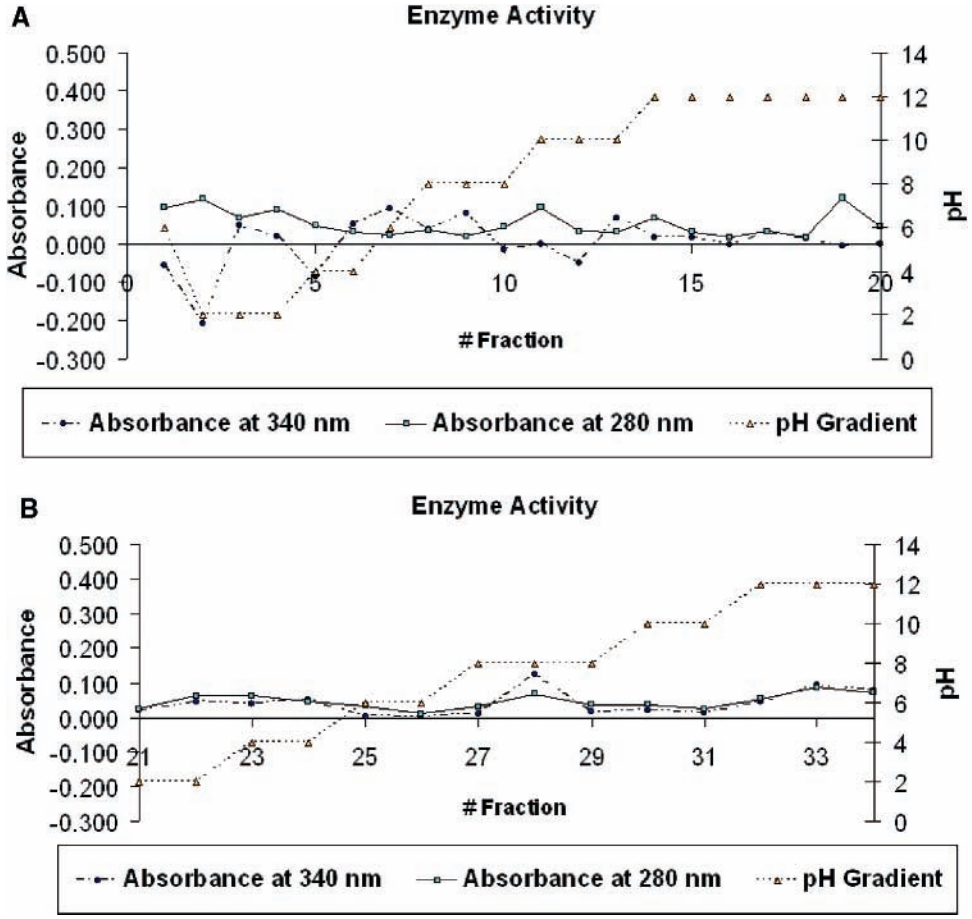

Figure 3. (A) Chromatograhic separation of alcohol dehydrogenase from *Methylobacterium extorquens*: (A) step 1 and (B) step 2.

The results for both the bacterium and the strawberry are shown in **Figures 3A**,**B** and **4A**,**B**. The fraction with the highest dehydrogenation activity from the second elution was used for further SDS−PAGE. Purification results for the bacterial and strawberry ADHs are shown in **Table 2**. Yield (%) is the percent fraction of the total activity (*U*) in purification step 1 toward the total activity (*U*) in purification step 2. Purification (fold) is the fraction of the specific activity [*U* (mg of protein)$^{-1}$] in purification step 1 toward the specific activity [*U* (mg of protein)$^{-1}$] in purification step 2.

SDS−PAGE. An aliquot of 40 μg of protein was loaded in each sample well. The results for SDS−PAGE are shown in **Figure 5A**. On the basis of these figures (lane 3), it is suggested that the molecular weight of the ADH from *Methylobacterium extorquens* is 45 kDa. The respective molecular weight of the ADH from *Fragaria* × *ananassa* (data obtained through regression analysis) is 24.6 kDa.

Native PAGE. An aliquot containing 40 μg of protein was loaded in each sample well. The results for native PAGE as follows: lane M, molecular weight markers; lane 1, ADH from *Methylobacterium* extorquens; lane 2, ADH from *Fragaria* × *ananassa*. Numbers on the left of molecular weight markers indicate the molecular weight of the proteins in kDa. (**Figure 5B**). From these data, it can be suggested that the molecular weight of the ADH from *Methylobacterium extorquens* is 45 kDa. The respective molecular weight of the ADH from *Fragaria* × *ananassa* (data obtained through regression analysis, data not shown) is 25 kDa.

According to the electrophoresis results (**Figure 5A**,**B**), the native and reduced forms of the enzymes exhibit similar molecular weights. This suggests that both the bacterial and the plant ADH(s) exist as a single polypeptide chain.

Activity of Extracted Proteins from Native Electrophoresis Gel. The activities for the extracted proteins (ADH) were found to be 24.74% for the bacterium and 30.09% for the strawberry. The percentage value refers to the ratio of the ADH activity before electrophoresis to that after electrophoresis.

Growth Curve of *M. extorquens* and the Biotransformation of Lactaldehyde. The growth of *M. extorquens* in a medium containing 0.75% (v/v) 1,2-propanediol, 0.25% (v/v) methanol, and 1% (w/v) peptone as a nitrogen source (this mixture is defined as the standard) and the formation of lactaldehyde were screened over the course of 10 days (**Figure 6**).

The bacteria followed a rather typical exponential growth phase followed by a stationary growth phase. The exponential growth from day 2 to day 4 was rather sharp. The maximum levels of lactaldehyde (3517 ppm) were obtained when the bacterial growth had reached the stationary phase (day 7). After this day, the levels of lactaldehyde were decreasing (2912 ppm on day 10).

Effect of 1,2-Propanediol in the Presence of Methanol. In this series of experiments, the effect of different levels of 1,2-propanediol on the bacterial growth was studied while keeping the levels of methanol constant in all experiments [0.25% (v/v)]. Because of the sharp exponential growth that had been observed, the bacterial growth in this series of experiments was studied for a time course of 96 h (**Figure 7**A). In addition to the standard run (i.e., 0.75% 1,2-propanediol), three different levels of 1,2-propanediol were used: 0.1%, 0.5%, and 1% (v/v). On day 7, the cells were centrifuged, and the levels of lactaldehyde were determined in the supernatant (**Table 3**). This

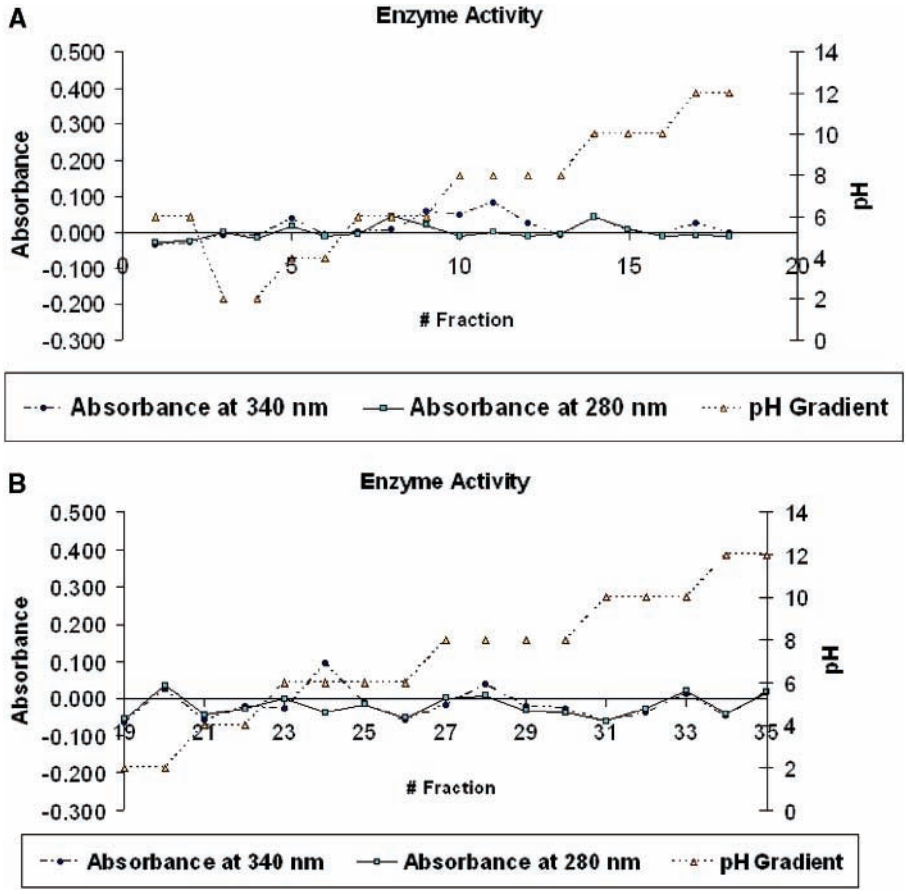

Figure 4. Chromatograhic separation of alcohol dehydrogenase from *Fragaria* × *ananassa*: (A) step 1 and (B) step 2.

Table 2. Purification (Fold) for *Methylobacterium extorquens* and *Fragaria* × *ananassa*

step	total protein (mg)	total activity (*U*)	specific activity [*U* (mg of protein)$^{-1}$]	yield[a] (%)	purification (fold)[b]
		A. Methylobacterium extorquens			
1	268.2	226.7	0.8	100	1.0
2	76.3	178.5	2.3	79	2.8
		B. *Fragaria* × *ananassa*			
1	185.5	200.2	1.1	100	1.0
2	68.3	171.2	2.5	86	2.3

[a] Yield (%) is the percent fraction of the total activity (*U*) in purification step 1 toward the total activity (*U*) in purification step 2. [b] Purification (fold) is the fraction of the specific activity [*U* (mg protein)$^{-1}$] in purification step 1 toward the specific activity [*U* (mg protein)$^{-1}$] in purification step 2.

result suggests that 1,2-propanediol is involved in the primary metabolism of *M. extorquens* and that the role of the diol is not only confined to the production of lactaldehyde. At day 7, bacterial cultures were analyzed for lactaldehyde (Table 3). In control runs, where 1% methanol but no 1,2-propanediol was used, lactaldehyde was not detected, as previously reported (*9*), The results for the standard run were very close to those observed in the previous series (about 3500 ppm). The highest levels of lactaldehyde (77% higher than the standard run) were found for the run using 0.5% (v/v) of 1,2-propanediol followed by the run using 1% (v/v) of 1,2-propanediol (17% higher than the standard run).

Effect of 1,2-Propanediol in the Absence of Methanol. Given the facultative methylotrophy of *M. extorquens* (*9*), we examined whether the bacteria needed the presence of methanol to grow and produce the aldehyde or whether the diol could suffice as the necessary carbon source for the bacterial growth. *M. extorquens* was grown using three different levels of 1,2-propanediol (0.1%, 0.5%, and 1%) where 0.75% 1,2-propanediol and 0.25% methanol were used in the standard run (**Figure 7B**). Lactaldehyde was not detected in the cultures with 0.1% and 0.5% 1,2-propanediol, whereas the cultures with 1% 1,2-propanediol produced 103% higher levels of lactaldehyde than the standard cultures (**Table 3**). These results suggest that (a) methanol is involved in the bacterial growth as the bacteria grew fastest when methanol was present and (b) the optimum growth conditions are obtained when the methyl providing compound-(s) (either 1,2-propanediol on its own or 1,2-propanediol and methanol) are present in a (total) ratio of about 1%.

DISCUSSION

The role of PPFMs in the biosynthesis of strawberry flavor cannot be easily revealed because this type of plant−microbe interaction can be disguised as part of the plant activities, thus being observed as plant biochemical reactions and not bacterial ones. It has been demonstrated, however, that PPFMs can enhance the flavor biosynthesis in strawberry callus cultures (*9*), whereas the bacteria can find a niche in strawberry tissue by consuming the methyl alcohols of the plant (e.g., 1,2-propanediol) (*10*). These data suggested a possible relationship of the bacterial and plant cells; with the work presented, the two elements of this relationship (i.e., bacterial as opposed to plant ADH activities) are studied and compared.

In extracts of *M. extorquens*, an organism able to grow with 1,2-propanediol as an energy and carbon source, high NAD-dependent ADH activities were found with 1,2-propanediol and

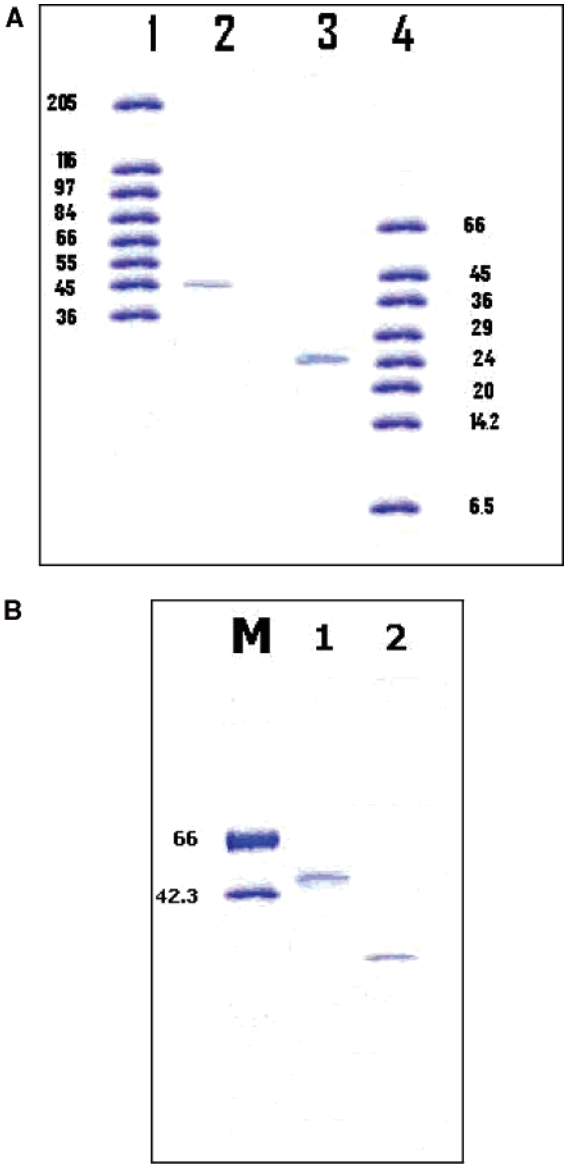

Figure 5. (A) Alcohol dehydrogenase characterization from *M. extorquens* and *F.* × *ananassa* by SDS–PAGE. Lane 1: Molecular weight markers (high range). Lane 2: Alcohol dehydrogenase from *Methylobacterium extorquens*. Lane 3: Alcohol dehydrogenase from *Fragaria* × *ananassa*. Lane 4: Molecular weight markers (low range). Numbers on the left and right of the molecular weight markers indicate the molecular weights of the marker proteins in kDa. (B) Alcohol dehydrogenase characterization from *M. extorquens* and *F.* × *ananassa* by native PAGE. Lane M: Molecular weight markers. Lane 1: Alcohol dehydrogenase from *Methylobacterium extorquens*. Lane 2: Alcohol dehydrogenase from *Fragaria* × *ananassa*. Numbers on the left of molecular weight markers indicate the molecular weights of the proteins in kDa.

methanol as substrates. The best substrates for the bacterial ADH were methanol and 1,2-propanediol, whereas 1-propanol and ethanol were the best substrates for the strawberry ADH. No significant ADH activity was observed for formaldehyde with the strawberry's enzyme, whereas there was significant ADH activity for the bacterium. From these results, it can be deduced that both the bacterial and plant ADHs have a tendency for alcohols with shorter carbon chains (i.e., alcohols with up to three carbon atoms). In addition, the bacteria have a much higher affinity for small alcohols than the plant ADH; this affinity could contribute to the formation of short-chain aldehydes possibly contributing to the flavor of strawberries.

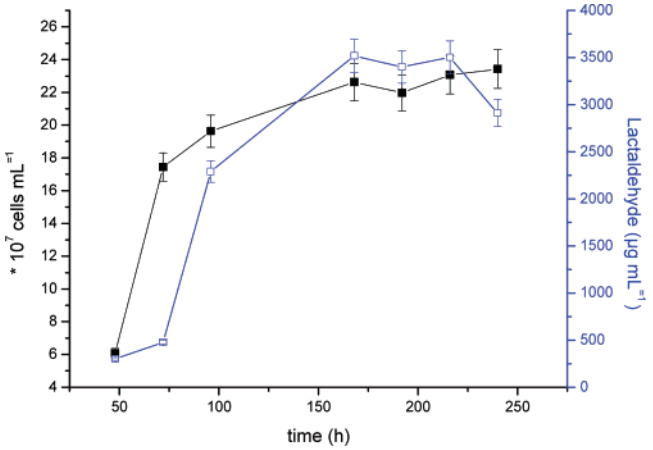

Figure 6. *Methylobacterium extorquens* growth and the biotransformation of lactaldehyde (□, concentration of lactaldehyde; ■, cell counts). The error bars correspond to replicate measurements.

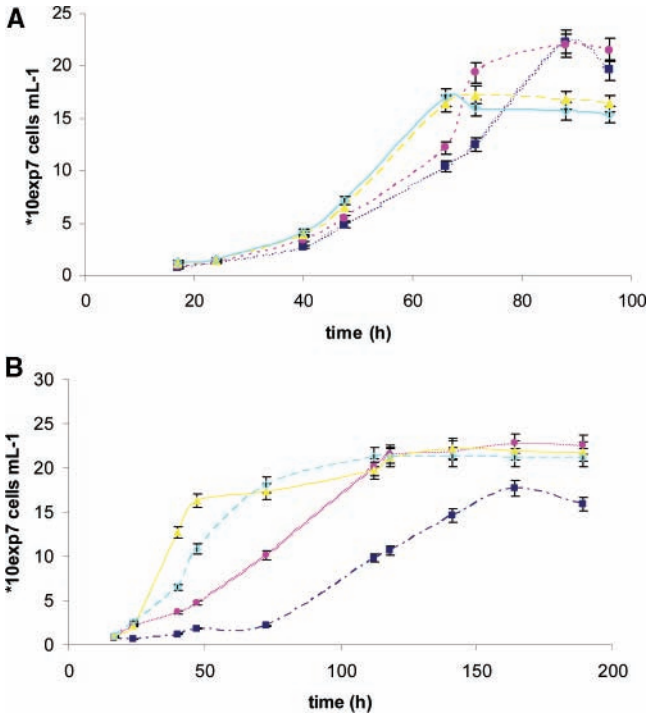

Figure 7. *Methylobacterium extorquens* growth in media containing 0.1% (■), 0.5% (●), 0.75% (▲), and 1% (◇) 1,2-propanediol (A) with 0.25% methanol and (B) in the absence of methanol. The error bars correspond to replicate measurements.

Table 3. Levels of Lactaldehyde at Day 7 When *Methylobacterium extorquens* Was Grown with Various Levels of 1,2-Propanediol Either with or without Methanol Present[a]

level of 1,2-propanediol	with methanol (0.25% v/v)	without methanol
0.1% (v/v)	1310 ± 103	n.d.[b]
0.5% (v/v)	6200 ± 345	nn.d.[b]
0.75% (v/v)[c]	3500 ± 145	3400 ± 128
1% (v/v)	4097 ± 189	6900 ± 295

[a] Values are presented as the average (standard deviation of triplicate runs. [b] n.d.: not detected. [c] Standard.

Upon conversion of the K_m values to percent K_m values, Figure 8 is obtained. It is thus suggested that there is a tendency for the bacterium to have a preference for alcohols with three

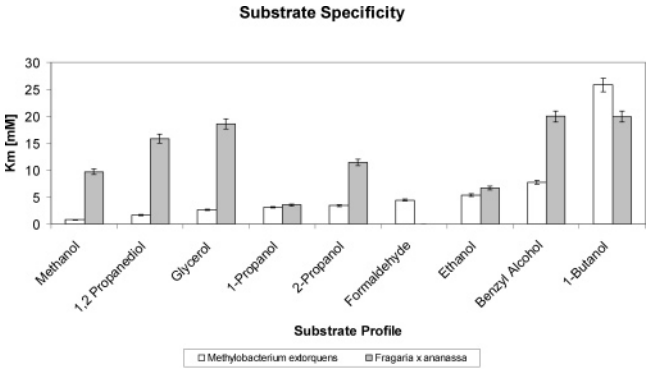

Figure 8. Relative percent K_m values. The error bars correspond to replicate measurements. For the bacterium, the K_m value of methanol is divided with all the K_m values of the other alcohols and the final result is expressed in (%) percentage activity. For the strawberry the K_m value of 1-propanol is divided by all of the K_m values of the other alcohols, and the final result is expressed in percentage activity. The error bars correspond to replicate measurements.

carbon atoms and more than one hydroxyl group (e.g., 1,2-propanediol and glycerol) whereas the strawberry's enzyme has a better affinity for alcohols with one hydroxyl group (e.g., 1-propanol and ethanol). It was also found that the bacterial ADH has a much higher affinity than the plant ADH for all of the substrates tested except from 1-butanol. This high affinity could greatly contribute to the generation of short-chain aldehydes by the bacterial oxidation of the strawberry-derived alcohols, in a way similar to that of the bacterial bioformation of lactaldehyde from the strawberry-derived 1,2-propanediol (Figure 6). The bacterial enzymatic activity with its affinity for a range of substrates could also contribute to the bioformation of a wide range of aldehydes that are flavor components in strawberry. It could thus be suggested that there is no genetic need for the plant to genetically code many different ADH activities, but rather a small number of ADHs could probably generate the whole spectrum of aldehydes and acids that contribute to the strawberry flavor.

Another point that can be deduced from our data is that the K_m value for the bacterial ADH with 1,2-propanediol is 9.6 times smaller than the respective strawberry ADH, a fact that implies the close relationship and the greater affinity−specificity for this compound by the bacterium. Therefore, an enzymatic collaboration between the bacterium and the strawberry in the biosynthesis of lactaldehyde is suggested here.

Apart from the affinity comparisons, we also purified the ADHs, obtaining purifications of 2.8-fold for the bacterium ADH and 2.3-fold for the strawberry ADH. The magnitudes of the purifications obtained here are similar to the reported value for the ADH of *Desulfovibrio gigas*, for which a result of 3.0-fold for the amount of the enzyme's purification was reported (*24*).

The ADH enzymatic activity was also observed after native electrophoresis and extraction of the protein bands. It can thus be concluded that the obtained purified bands after SDS electrophoresis correspond to ADH enzymes.

ADHs contain a metal in their active center—usually Zn (*20*)—but they are also strongly inhibited by Zn^{2+}, as well as by other metals (*20*, *24*). The main reason for the selection of these metal ions is the fact that some of them can be found in plant tissues and can participate in the regulation of some of the plant activities. The zinc ion is used to attract the approaching alcohol through polarization of its oxygen (in hydroxyl, −OH) atom (*20*). From the presented data, it is clear that certain metals—especially heavy metals—cause a certain amount of deactivation to the enzyme for both the bacterium and the strawberry itself. The fact that EDTA reduces the enzymatic activity, for both the bacterium and the strawberry, is additional evidence that the enzyme either contains a metal in its active center or is activated by metal ions that are blocked by the chelate action.

From the SDS−PAGE results, it could be suggested that there is a significant difference in the values of the molecular weights of the two ADHs (20.4 kDa difference), or in other words, the molecular weight of the strawberry enzyme is 55% of that of the bacterial enzyme. The molecular weight of an oxygen-labile, NAD-dependent alcohol dehydrogenase from *Desulfovibrio gigas* was reported to be approximately 49 kDa (*24*).

Our study on the pH effect on the bacterial activity has shown that ADH activity is highest at pH 6, which is in good accord with similar studies on methylotrophic bacterial enzymes (*2*). However, only 40% of this maximum activity was observed at pH 4, which is the pH of the strawberry fruit.

It is also noticeable that not only *M. extorquens* but also other bacteria such as *Bacillus megaterium*, which can also be found in strawberries, can participate in the formation of chemicals that are useful for plant interactions such as acroleins (*25*). Our current work focuses on the interactions of bacterial (*M. extorquens*) and strawberry (*Fragaria* × *ananassa* cv. Elsanta) cells to further highlight the role of the bacteria in the biosynthesis of DMHF.

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