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The formation of 2,5-dimethyl-4-hydroxy-2*H*-furan-3-one by cell-free extracts of *Methylobacterium extorquens* and strawberry (*Fragaria* × *ananassa* cv. Elsanta)

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Abstract

The formation of 2,5-dimethyl-4-hydroxy-2*H*-furan-3-one (DMHF), using the bacterial cells of *Methylobacterium extorquens* (strain with CABI registration number: IMI 369321) and strawberry cells (*Fragaria* × *ananassa*cv. Elsanta), was studied. A combined mixture of the bacterial cell-free extract plus the strawberry extract was used as the enzymatic source. The pH of all the experimental aliquots was pH 6.0. The studied substrates were short-chain alcohols, carbohydrates and one amino acid. DMHF was analyzed by HPLC with UV detection at 280 nm. When 1,2-propanediol, 1-propanol, sucrose and fructose were used as substrates, the best production rates of DMHF were achieved, having a combined mixture of the bacterium and strawberry (minus the achenes) as the enzymic source. On the other hand, glucose, 1,2-propanediol, 2-propanol and fructose were the best substrates for the production of DMHF when a combined mixture of the bacterium and achenes served as the enzymic source. All results were obtained by comparing the production of DMHF from the strawberry extract alone and the bacterium plus the strawberry, without the achenes (in the first case) and the production of DMHF from the achene extract alone and the bacterium plus the achenes (in the second case). Moreover, the dehydrogenation activities of these three enzyme sources were determined by measuring the absorption of NADH at 340 nm. The substrate which was used for this series of the experiments was 1,2-propanediol. These results indicate the undoubted cooperation between the plant and the bacterial cells. This paper reports, for the first time, the formation of DMHF by bacterial and plant cell-free extracts.

Keywords: Flavour; 2,5-Dimethyl-4-hydroxy-2*H*-furan-3-one (DMHF); Achenes; Strawberry; *Methylobacterium extorquens*

1. Introduction

Methylotrophic bacteria and their relationships with the host plants have been subjects of interest, from both theoretical and practical points of view, for a significant number of years (Holland & Polacco, 1994). The interest in methylotrophy, regarding the food industry, began to rise since new applications of methylotrophs have been demonstrated (Anthony, 1982). Moreover, microorganisms that live together with plants represent a huge majority of the total microbe population. Methylotrophic bacteria are involved in many interesting *in vivo* interactions between plants and bacteria. The species of pink pigmented facultative methylotrophs (PPFMs) is biologically very significant because of its intimate relationship with the plant (Holland & Polacco, 1994).

One of the most important components of strawberry flavour is 2,5-dimethyl-4-hydroxy-2*H*-furan-3-one (DMHF) (Bood & Zabetakis, 2002). The synthesis of DMHF in strawberry, as well as its synthesis performed by microorganisms, has been studied by various research groups over the past decade (Perez, Olias, Sanz, & Olias, 1996; Zabetakis & Holden, 1996; Hequet, Sancelme, Bolte, & Demuynck, 1996). Fructose 1,6-biphosphate was found to be a precursor molecule of DMHF (Roscher, Bringmann, Schreier, & Schwab,

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1998). However, little research has been done on the origin of earlier precursors of DMHF in strawberry, where our study mainly focuses.

Because of the significance, both as a chemical compound and a biosynthetic precursor of lactaldehyde (2-hydroxypropanal) (Zabetakis, Moutevelis-Minakakis, & Gramshaw, 1999), and the growing interest in biochemistry of *Methylobacterium extorquens*, we report here the conversion of a variety of precursor molecules – carbohydrates, alcohols and one amino acid – to DMHF in cell-free extracts of *M. extorquens* and strawberry extracts. A possible co-operation between strawberry and *M. extorquens* has been suggested (Zabetakis, 1997), regarding their enzyme systems which affect the dehydrogenation (oxidation) of certain alcohols. Alcohol dehydrogenation activity of the aforementioned bacterium and the strawberry has been reported (Koutsompogeras, Kyriacou, & Zabetakis, 2006). The elucidation of this collaboration and the extent of it, would lead us to conclusions about the way the strawberry and the bacterium communicate, benefit from each other and collaborate in the strawberry flavour synthesis. Furthermore, a biotechnological application for the production of DMHF may arise from the collaboration of the bacterium and the strawberry enzyme system and cells, thus producing a commercial product in an innovative and commercial way.

2. Materials and methods

2.1. Chemicals

All chemicals used were of the highest purity commercially available by: Chemicals Merck (Darmstadt, Germany), Sigma Aldrich (Saint Luis, USA) and Fluka (Buchs, Switzerland).

2.2. Bacterial cultivation and cell-free extract preparations

M. extorquens (strain with CABI registration number IMI 369321), was isolated from strawberry callus (Zabetakis, 1997) and 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 previously described (Zabetakis, 1997). Cells were harvested at the end of their exponential phase then centrifuged (Gallenkamp centrifuge) at 9000*g* for 10 min, washed twice with 0.1 M phosphate buffer (pH 8.0), recentrifuged (9000*g* for 10 min) and stored in 50 ml of the same buffer; 2 g of the bacteria were subsequently dissolved in 50 ml phosphate buffer (0.2 M pH = 8.0) and cells were broken by ultrasonic disintegration (Branson Ultra sound device) under ice-cooling. The cell-free extract which was used for enzyme assay was stored at 2 °C. This solution also included 2% (w/v) soluble polyvinylpyrrolidone (PVP) as a phenolic scavenger, 1 mM $ZnSO_4$, 10 mM β-mercaptoethanol (BME) as a reducing agent, and the following protease inhibitors: 0.8 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine hydrochloride (BHC), and 5 mM amino-*n*-caproic acid (ACA).

The enzyme solution from strawberries was produced by placing 200 g of strawberries (*Fragaria* × *ananassa*) in an OMNI Mixer Speed 2 programme, for 10 min. The pulp was then filtered under vacuum (Whatman #401 filter paper) and the filtrate was stored at 2 °C. This filtrate also contained 2% (w/v) soluble polyvinylpyrrolidone (PVP) as a phenolic scavenger, 1 mM $ZnSO_4$, 10 mM β-mercaptoethanol (BME) as a reducing agent, and the following protease inhibitors: 0.8 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine hydrochloride (BHC), and 5 mM amino-*n*-caproic acid (ACA). Seeds (achenes) were removed from ripe strawberries, washed with distilled water, then placed on a Whatman chromatography paper (No. 3) and were air-dried . Seeds were ground in 1.5 ml microfuge tubes using a glass tissue grinder in a chilled (4 °C) extraction buffer (in the ratio of 1 ml of buffer per 100 mg of seeds). The composition of the extraction buffer was 100 mM Tris–HCl (pH 7.5) including 2% w/v soluble polyvinylpyrrolidone (PVP) as a phenolic scavenger, 1 mM $ZnSO_4$, 10 mM mercaptoethanol as a reducing agent, and the following protease inhibitors: 0.8 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM α-amino-*n*-caproic acid (ACA). The extract was centrifuged at 13,600*g* for 15 min and the supernatant was collected as a crude enzyme source.

2.3. Enzyme assays

The enzyme assays were based on measuring the absorption at 340 nm for NADH (Georgatsos, 1991). NAD (as sodium salt) was used as an electron acceptor. The reaction was initiated by adding the substrate. The spectrophotometer which was used was a Helios *β* Model. 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 6 different fruits. The bacterial alcohol dehydrogenase (ADH) activities were assessed and the optimum pH (results not shown) for ADH activity was found to be pH 6.0. All of the experiments were conducted at this pH. The standard dehydrogenation assays contained: 35 mM phosphate buffer,1 mM NAD, 0.1 ml enzyme extract (either from the bacterium or strawberry, respectively), in a total volume of 3 ml. The reaction was started by injecting the respective alcohol each time. The incubation time was 10 min while the material used for the measurements were disposable spectrophotometer plastic cells having a diameter of 1 cm.

2.4. HPLC analysis

The HPLC device which was used was an Agilent 1100 Series. The mobile phase was a 90% v/v 0.2 M sodium acetate buffer solution, with 10% v/v methanol. The mobile

phase was initially filtered through a Whatman #1 filter paper. The flow rate was set to be 1.0 ml/min while the HPLC column was a Zorbax C – 18 (4.6 × 150 mm) Agilent chromatographic column. In order to clarify the solution from proteins and other interfering molecules, the following procedure was followed: 2.5 ml of the raw sample were mixed with 125 μl of Carrez I solution (15% w/v $K_4Fe(CN)_6$) plus 125 μl of Carrez II (30% w/w $ZnSO_4$) solution. The final product was left to settle and then the supernatant was filtered through a Whatman #1 filter paper and a 40 μm microfilter of the sample after the Whatman filtration; 20 μl of the filtrate were injected to the HPLC system. UV detection was carried out at 280 nm. In order to make qualitative calculations, a linear range of concentrations of standard (DMHF) was made in the range 0.5–42.0 ppm. Based on the HPLC result, the equation of the standard curve is the following:

$$Y = -0.0525 + 2.1 \times 10^{-6} X, \quad (1)$$

where Y is the concentration of DMHF in ppm and X is the area of the HPLC peak, with $R^2 = 0.9992$.

2.5. Kinetics of 1,2-propanediol conversion

The production of DMHF, with the use of 1,2-propanediol as substrate, was studied in these series of experiments. Two series of experiments were designed and performed. The first experiment contained only the strawberry enzymes and the second one contained a mixture of the strawberry and the bacterial enzymes. The samples (in a total volume of 5 ml) contained 1,2-propanediol (100 mM), 1 ml of the strawberry lysate and phosphate buffer (100 mM, pH 6.0) for the strawberry experiment. For the combined experiment, the same procedure as above was followed but the combined mixture also contained 1 ml of the bacterial cell-free extract. All the samples were prepared as triplicates. A third row of experiments was prepared in the same way with the strawberry experiment but this time, instead of the strawberry enzyme system, the bacterial cell-free extract was used. The samples were incubated for a period of 4 days at 30 °C in a water bath. Samples were taken every 12 h, stored in a freezer and analysed at the end of the 5th day. All the samples were analysed by the procedure described in Section 2.4. The samples were prepared as described in Section 2.3. This time, though, a wider variety of precursor molecules was chosen (each at concentration of 100 mM). The 12 precursors were: 1-propanol, 1,2-propanediol, 2-propanol, fructose, galactose, glucose, glutamic acid, glycerol, mannitol, methanol, rhamnose and sucrose. The test tubes containing the samples described before were incubated at 30 °C in a water bath. All the samples were analysed by the procedure described in Section 2.4.

3. Results and discussion

3.1. Comparison of the dehydrogenation activities between strawberry and bacterial cell-free extract

The substrate which was used in all the following experiments was 1,2-propanediol. Methylobacterium (*M*), is the dehydrogenation activity of the cell-free extract of the bacterium measured at 340 nm for the production of NADH. Strawberry (S), is the dehydrogenation activity of the extract of the strawberry (without achenes) measured at 340 nm. Achenes (A), is the dehydrogenation activity of the cell-free extract of the strawberry acehenes measured at 340 nm. We define, as experimental sum [B + S + A], the dehydrogenation activity of the extract of the strawberry (body and achenes) plus the cell-free extract of the bacterium measured at 340 nm while the theoretical sum [B + S + A],is calculated as the algebraic sum of (M) + (S) + (A). The results from all the spectrophotometric analyses are shown in Figs. 1–4.

When the dehydrogenation activities of the bacterium [B] and the strawberry [S] were compared (Fig. 1), it was

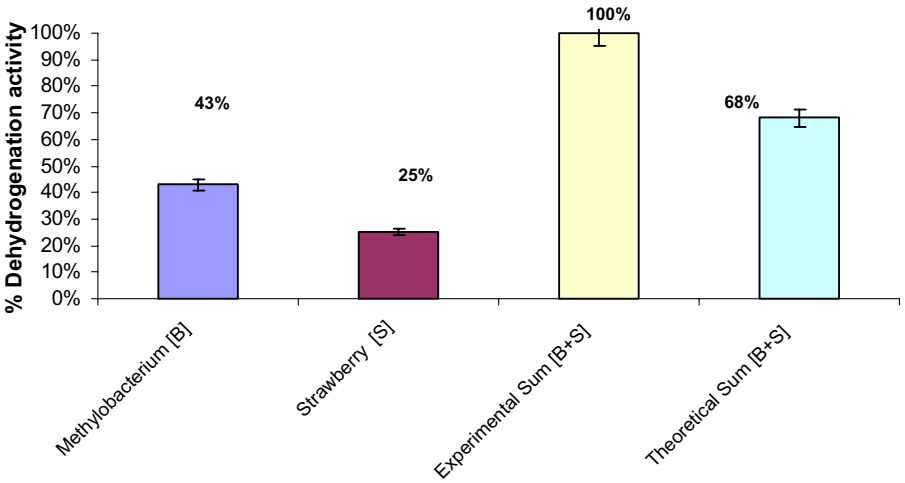

Fig. 1. The dehydrogenation activities of methylobacterium and strawberry cell-free extracts along with their theoretical and experimental sums. The value of 100% corresponds to the experimental sum of the dehydrogenation activity of both sources. The substrate used was 1,2-propanediol. The error bars correspond to replicate measurements.

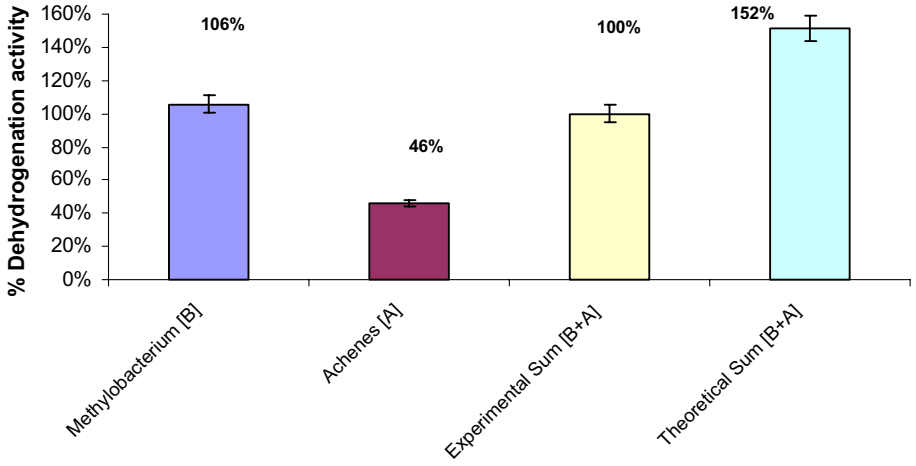

Fig. 2. The dehydrogenation activities of methylobacterium and achenes and cell-free extracts, along with their theoretical and experimental sums. The value of 100% corresponds to the experimental sum of the dehydrogenation activity of both sources. The substrate used was 1,2-propanediol. The error bars correspond to replicate measurements.

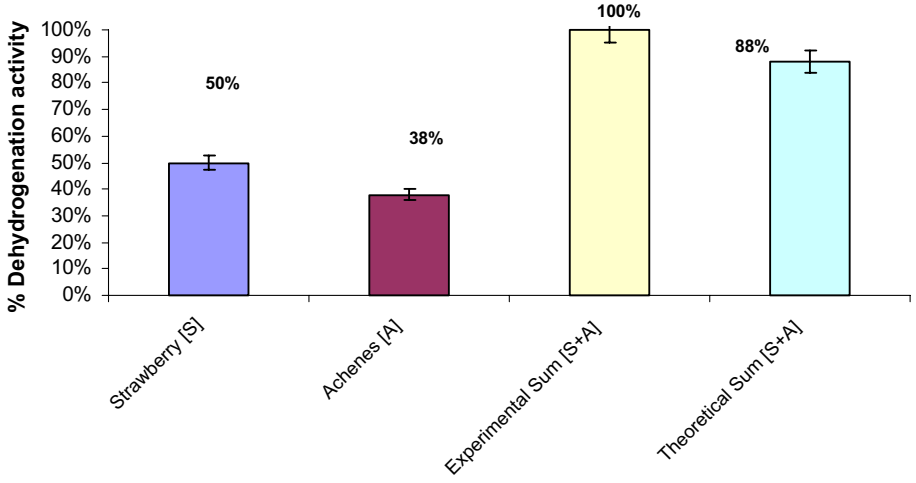

Fig. 3. The dehydrogenation activities of strawberries and achenes cell-free extracts, along with their theoretical and experimental sums. The value of 100% corresponds to the experimental sum of the dehydrogenation activity of both sources. The substrate used was 1,2-propanediol. The error bars correspond to replicate measurements.

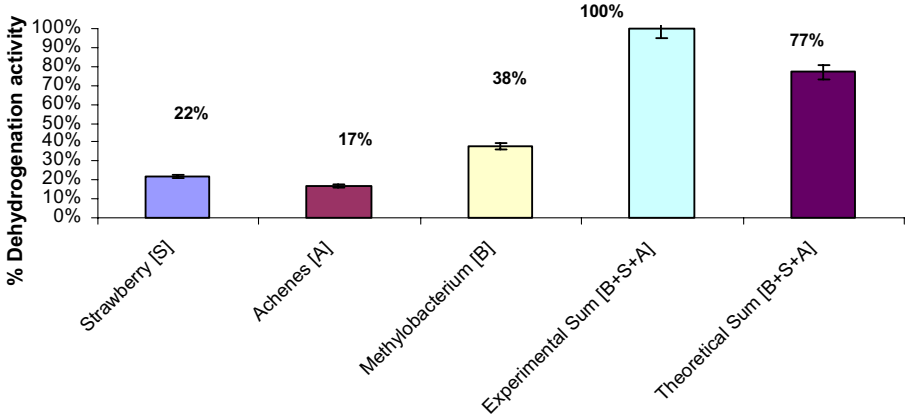

Fig. 4. The dehydrogenation activities of methylobacterium, strawberries and achenes cell-free extracts, along with their theoretical and experimental sums. The value of 100% corresponds to the experimental sum of the dehydrogenation activity of the three sources. The substrate used was 1,2-propanediol. The error bars correspond to replicate measurements.

concluded that the enzymic activity of the methylobacterium corresponded to the 43% of the experimental sum of the dehydrogenation activity of the combined mixture. Moreover, the ADH activity of strawberries corresponded to 25% of the experimental sum of the dehydrogenation activity of the combined mixture. Furthermore, the theoretical sum of the dehydrogenation activity corresponded to (43 + 25=) 68% of the experimental measured value.

When the dehydrogenation activities of the bacterium [B] and the achenes [A] were compared (Fig. 2), it was observed that the enzymic activity of the methylobacterium corresponded to 106% of the experimental sum of the dehydrogenation activity of the combined mixture. Moreover, the ADH activity of achenes corresponded to 46% of the experimental sum of the dehydrogenation activity of the combined mixture. Furthermore, the theoretical sum of the dehydrogenation activity corresponded to 152% of the experimental value.

When the dehydrogenation activities of the strawberry [S] and achenes [A] were compared (Fig. 3), it was concluded that the enzymic activity of the strawberry corresponded to 50% of the experimental sum of the dehydrogenation activity of the combined mixture. Moreover, the ADH activity of achenes corresponded to 36% of the experimental sum of the dehydrogenation activity of the combined mixture. Furthermore, the theoretical sum of the dehydrogenation activity corresponded to (50 + 38=) 88% of the experimental measured value.

When the dehydrogenation activities of methylobacterium [B] plus the strawberry [S] and achenes [A] were compared (Fig. 4), it was concluded that the enzymic activity of the Methylobacterium corresponded to 38% of the experimental sum of the dehydrogenation activity of the combined mixture. Moreover, the ADH activity of achenes corresponded to 17% of the experimental sum of the dehydrogenation activity of the combined mixture. Additionally, the ADH activity of strawberries corresponded to 22% of the experimental sum of the dehydrogenation activity of the combined mixture. Furthermore, the theoretical sum of the dehydrogenation activity corresponded to (38 + 17 + 22=) 77% of the experimental measured value.

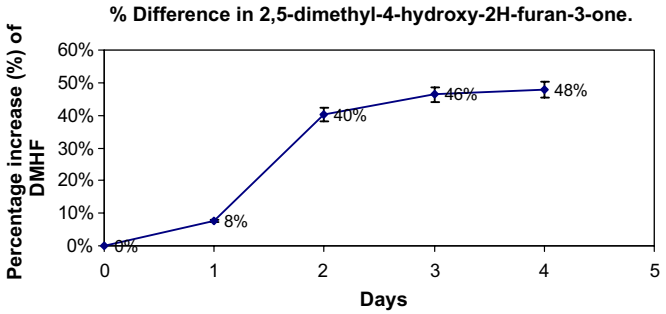

Fig. 5. Percentage increase (%) of the produced 2,5-dimethyl-4-hydroxy-2*H*-furan-3-one (DMHF) when 1,2-propanediol was used as substrate. The comparison is based on the production of DMHF between the strawberry enzymes alone and the combined mixture of the Methylobacterium and the strawberry. The error bars correspond to replicate measurements.

3.2. HPLC analysis – kinetics of 1,2-propanediol conversion

All the subsequent calculations for DMHF were based on Eq. (1). The HPLC results on the kinetics of 1,2-propanediol conversion to DMHF are shown in Fig. 5.

The percentage increase (%) refers to the difference calculated between the strawberry and the mixture of strawberry and bacterial cell-free extract. The results referring to the sample of the fifth day are not shown. It is important to note that there was no production of DMHF from the sample which contained the bacterial cell-free extract alone. From the results of this graph, it was decided to incubate all the precursors in the same way as with 1,2-propanediol and to quantify the DMHF produced at the end of the fourth day.

3.3. Precursor incubation

The samples were prepared and analysed as described in Section 3.2 and the results are shown in (Fig. 6). For the

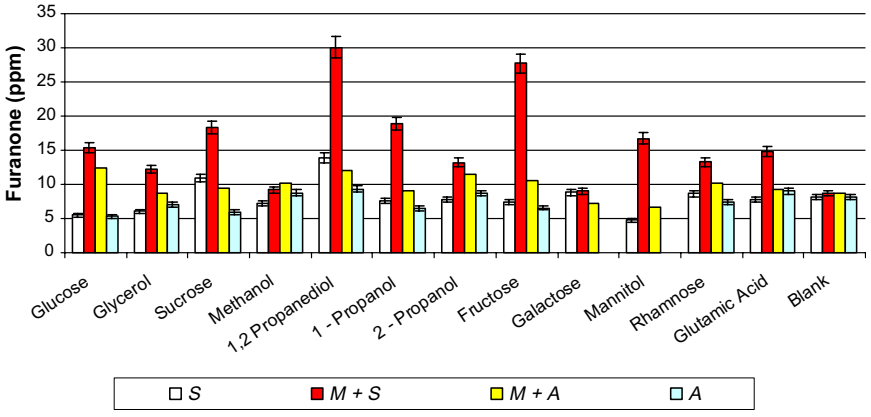

Fig. 6. The levels of 2,5-dimethyl-4-hydroxy-2*H*-furan-3-one (DMHF) produced by the cell-free extracts of strawberry [S] (white bars), strawberry with bacteria [M + S] (red bars), achenes [A] (blue bars), achenes with bacteria [M + A] (yellow bars). The error bars correspond to replicate measurements. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

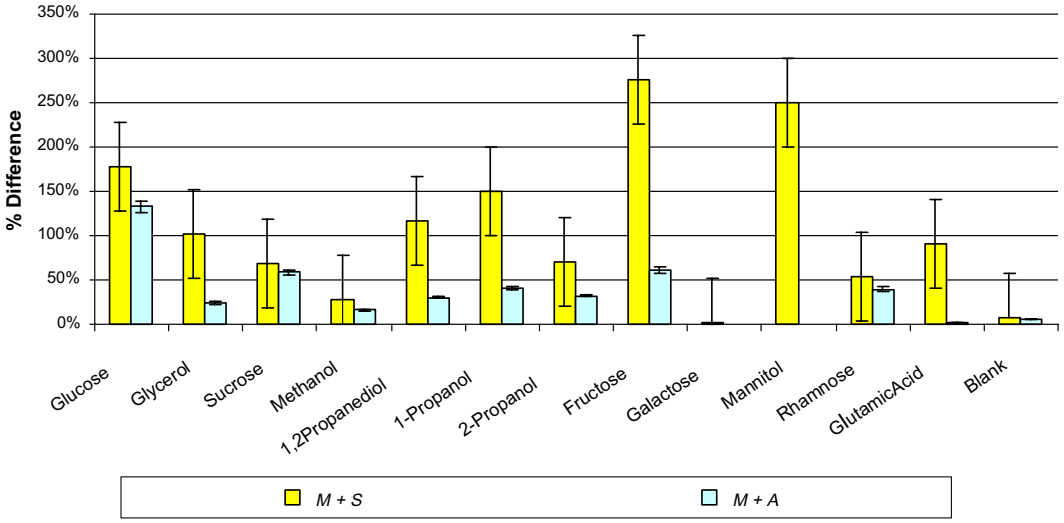

Fig. 7. Comparative values in the production of DMHF (% difference) between strawberry with bacteria [M +S] and achenes with bacteria [M + A]. The error bars correspond to replicate measurements.

methylobacterium and strawberry mixture, it can be seen that the best substrates are 1,2-propanediol, 1-propanol, sucrose and fructose for the production of DMHF (absolute values). On the other hand, when the absolute values of concentration were converted into comparative ones, between the bacterium and the strawberry (% difference), it can be seen that the best substrates were: glucose (177%), 1-propanol (149%), fructose (276%) and mannitol (250%) (Fig. 7).

For the mixture of methylobacterium and achenes, it can be suggested that the best substrates for the production of DMHF are glucose, 1,2-propanediol, 2-propanol and fructose (absolute values). On the other hand, if the absolute values of concentration are converted into comparative ones, between the bacterium and the strawberry (% difference), the best substrates are: 1-propanol (40%), sucrose (58%), fructose (61%) and glucose (133%) (Fig. 7).

These results indicate the undoubted cooperation between the plant and the bacterial cells. Numbers in parentheses indicate the percentage increase of the levels of DMHF. These data were calculated from the results of (Fig. 6). As was also pointed out in the previous section, there was no production of DMHF from the sample which only contained the bacterial cell-free extract.

In extracts of *M. extorquens*, an organism able to grow with 1,2-propanediol as energy and carbon source, high NAD-dependent ADH activities were found with 1,2-propanediol, as substrate (Koutsompogeras et al., 2006). Our group has reported the presence of 1,2-propanediol in strawberries (Zabetakis & Gramshaw, 1998) and *M. extorquens*, which can grow on them (Zabetakis, 1997) and can participate in a symbiotic relationship with the fruits (Holland et al., 1994; Zabetakis, 1997; Koutsompogeras et al., 2006).

Our current research is focused on the oxidation of 1,2-propanediol (Zabetakis & Gramshaw, 1998) and other strawberry-derived alcohols to the corresponding aldehydes. The results reported here suggest a combined enzymatic collaboration between the bacterium and the strawberry, leading to the formation of DMHF. The initial alcohols are converted into the respective aldehydes with the aid of enzymes from *M. extorquens*, which in turn could be transformed into DMHF with the use of enzymes from *Fragaria* × *ananassa*.

The best precursors for the production of DMHF in the combined mixture – compared to the production of DMHF in strawberries extracts alone – were found to be 1-propanol, 1,2-propanediol, fructose, sucrose and mannitol. This suggests that the enzyme system responsible for the production of DMHF requires or has a preference for hydroxyl-compounds such as carbohydrates (disaccharides or monosacharides) or short-chain alcohols, preferably with three carbon atoms. The bacterial enzymatic activity, with its affinity for a range of substrates, could also contribute to the formation of a wide range of aldehydes that are flavour components in strawberry. Fructose and sucrose may be converted into DMHF through different oxidation steps from 1,2-propanediol (Raab et al., 2006), even though, in the beginning, there may be some common dehydrogenation reactions.

There are many roles attributed to ADH, such as survival periods of hypoxia, regulation of growth, protection from freezing and biogenesis of volatiles of fragrance and flavour (Mitchell & Jelenkovic, 1995). A comparison of the oxidative capacities of the respective enzymes (ADH), concerning the bacterial and the strawberry cells, revealed that the combination of their dehydrogenation activities yielded a higher activity than did the theoretical sum of their individual ones. The same comparison of the oxidative capacities of ADH, when applied to the bacterium and achenes revealed that the combination of their dehydrogenation activities yielded a lower activity than the theoretical sum of their individual ones. Based on the same way of thinking, comparison of the oxidative capacities

of ADH, concerning the strawberry and achenes, revealed that the combination of their dehydrogenation activities yielded a higher activity than did the theoretical sum of their individual ones. Finally, the comparison of the oxidative capacities of ADH, concerning the enzymatic mixture of bacteria, strawberry and achenes revealed that the combination of their dehydrogenation activities yielded a higher activity than did the theoretical sum of their individual activities.

All of these data imply that the combined mixture of the enzymes from the three different sources reacts at a higher rate when they are all present together thus enhancing the metabolic pathway for the formation of fragrance compounds, such as DMHF in this study. In addition, the ADH activities from bacterium and strawberry tend to have a better cooperation when they are present together at the same time. The same conclusion applies for the ADH from achenes and strawberry. On the other hand, the cooperation of ADHs from bacterium and achenes is weakened when the two sources of enzymes are present together.

The best substrate for the NAD-dependent bacterial ADH is 1,2-propanediol, while 1-propanol is the best substrate for the strawberry ADH (Koutsompogeras et al., 2006). This fact, combined with the high DMHF production rates when these two cell-free extracts were used (Fig. 6), indicates a potential cooperation and correlation between the enzyme systems of the two sources regarding their contribution to the production of DMHF.

The ADH activity was determined in order to correlate the dehydrogenating activity with the formation of DMHF, since the first steps of these reactions may involve the participation of this enzyme. It has also been shown that DMHF can be synthesized from 1,2-propanediol (Zabetakis et al., 1999, Zabetakis, Gramshaw, & Robinson, 1999).

It has been proposed that the total amount of free amino-acids has a significant role in the formation of the strawberry flavour (Perez, Olias, Luaces, & Sanz, 2002; Perez, Olias, Sanz, & Olias, 1992). *Chandler* strawberries showed a 4–fold increase in ethyl esters over the duration of maturation, with a 10–fold reduction in alanine (Perez et al., 1992). Alanine was found to be the most important free amino-acid in the fruits, along with asparigine and glutamine (Perez et al., 1992). Accordingly, the metabolism of amino-acids is important in the formation of flavour in strawberries. The production of DMHF from glutamic acid in our experiments reinforced this (Perez et al., 1992) report. Carbohydrates have been proposed as precursor molecules of furanones, with fructose up to now the most likely candidate (Zabetakis & Holden, 1997; Sanz, Olias, & Perez, 1997; Zabetakis et al., 1999, 1999).

Our group has reported that the production of the glycoside of DMHF was activated by the addition 6-deoxy-D-fructose in the medium of strawberry tissue cultures (Zabetakis, 1997). The proposed pathway for the synthesis of volatile esters, another important group of compounds that participate in the flavour of strawberries, has been studied in the mature fruits of strawberries (Forney, Kalt, & Jordan, 2000). Accordingly, high activity of AAT leads to a higher production of esters and thus a stronger flavour.

Furthermore, research in the fields of genetic encoding of the various dehydrogenase enzymes in strawberries has recently shown that a 37 kDa, enone oxidoreductase, with an open reading frame consisting of 969 base pairs, is present in the parenchyma tissue of the strawberry fruit and is involved in the biosynthesis of DMHF (Raab et al., 2006). This is a very important finding and it demonstrates the importance of the oxidizing enzymes in strawberries and their potential biotechnological role. Our experimental results, showing that the bacterial cell-free extract cannot produce DMHF, when there are no strawberry extracts present, reinforce our suggestion that a co-operation between the bacterial and the plant cells occurs.

4. Conclusions

The formation of 2,5-dimethyl-4-hydroxy-2*H*-furan-3-one was found to be greater when a combined enzyme mixture of two cell-free extracts of *M. extorquens* and strawberry (*Fragaria* × *ananassa* cv. Elsanta) was used, compared to the enzyme system of the strawberries alone. The dehydrogenation activity of the combined enzyme mixture from the three different sources tends to produce a higher reaction rate when they are all present together and possibly enhances the formation of DMHF.

The flavour of strawberry is also very important in many manufactured food products. This is a matter of great significance and our current work focuses on the interactions of bacterial (*M. extorquens*) and strawberry (*Fragaria* × *ananassa* cv. Elsanta) cells in order to further highlight the role of the bacteria in the synthesis of DMHF. Moreover, a potential biotechnological application for the production of DMHF, by a combined action of the bacterium and the strawberry enzyme system is of possible significance.

Acknowledgements

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