

# Carbohydrate utilization of *Erwinia amylovora* in vitro

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**Summary:** Nectar is a multi-component aqueous solution that promotes bacterial multiplication. The concentration of nectar in plant flowers is not stable since it is under the influence of environmental conditions, especially free moisture and relative humidity. Experiments were conducted with "artificial nectar" and directed along two lines: (1) determination of the optimal concentrations of carbohydrates for the growth of *E. amylovora* development (2) consumption of different carbohydrates besides basic sugars.

Solutions of "artificial nectar" were prepared in different compositions by changing the dominance of basic sugars (fructose – glucose – sucrose) in proportions of 2:1:1, 1:2:1, 1:1:2 and between concentrations of 10–0.6% (diluted with Basal minimum broth) in order to determine optimal conditions for the development of *E. amylovora*.

At a basic sugar concentration of 10% bacterial multiplication started and continued until 1 log degree (from 10<sup>6</sup> to 10<sup>7</sup> cfu/ml). At concentrations of 5% and 2.5 % cells developed with nearly the same kinetics (from 10<sup>6</sup> to 8×10<sup>7</sup> cfu/ml and from 10<sup>6</sup> to 9×10<sup>7</sup> cfu/ml, respectively). Multiplication was more pronounced and nearly the same at concentrations of 1.2 % and 0.6 % (from 10<sup>6</sup> to 2×10<sup>8</sup> cfu/ml). At a basic sugar concentration 30% total sugars bacterial multiplication did not occur, while at 20 % it was negligible, not measurable photometrically.

At minimal concentrations of F, G, S (between 1–0.1 %) bacterial cells were still able to multiply, producing organic acids from sugars.

Our study showed that *E. amylovora* requires only a small amount of sugars (0.1%) for multiplication (acid production) while high concentrations inhibit multiplication. There was a negative correlation between sugar content and cell density. The optimal range of sugar concentration was at about 1%.

Effect of "less frequent carbohydrates" to *E. amylovora* multiplication was also determined using the API 50 CH strip. We could provide information on utilization of 39 carbohydrates by the bacterium at different categories as follows: Not utilized-, Slowly and weakly utilized-, Slowly and completely utilized-, Quickly and completely utilized carbohydrates. We suppose that carbohydrates that belong to the latter two groups could play an important role as nectar components in promoting *E. amylovora* multiplication in the blossoms of pome fruit trees.

**Key words:** API 50 CH strip, artificial nectar, fire blight, nectar, less frequent carbohydrates

## Introduction

Epidemics of fire blight incited by *Erwinia amylovora* (Burr.) Winslow et al. are extremely variable. The presence of high or low populations of bacteria in the host plant depends on blossom parts (Hasler & Mammig, 2001) as well as the histological structure of the nectary (Orosz-Kovács et al., 1991, 2002). Nectar is a multi-component aqueous solution that promotes bacterial multiplication. Nectar composition is a characteristic of plant species and even cultivars (Wykes, 1952, Kartashova & Novikova, 1961, Kapyla, 1978). The ratio of basic sugars, fructose (F), glucose (G), sucrose (S), as constituents of floral nectar was studied in various plant species (Baker & Baker, 1983, 1990). On the basis of nectar sugar composition, according to categories of Percival (1961), most apple cultivars belong to the „sucrose-rich" and „sucrose dominant" type (Orosz-Kovács et al. 1997, Nagy Tóth et al., 2000). Furthermore, according to them the secretory product of apple cultivars is balanced, and contains nearly equal amounts of fructose, glucose and sucrose. The nectar of pear, another fruit tree

species threatened by *E. amylovora*, consists mainly of glucose and fructose, whereas sucrose can be detected only in a few cultivars (Farkas et al., 2002). The concentration of nectar is under the influence of environmental conditions, especially free moisture and relative humidity (Hildebrand & Philips, 1936).

Nectar, getting to the surface through nectary stomata, acts as an attractant for pollinator insects, but also supports the epiphytic growth of bacteria. Conditions for the pathogen vary greatly, according to the time of nectar secretion, its sugar composition and concentration (Ivanoff & Keitt, 1941, Campbell et al., 1991, Johnson & Stockwell, 1998). The optimal sugar concentration for growth of *E. amylovora* ranges from 5 to 20% (Pusey, 1999).

Experiments were conducted with "artificial nectar" (sugar solutions of various composition, similar to natural nectars) and directed along two lines: (1) determination of the optimal concentrations of carbohydrates for growth of *E. amylovora* (2) consumption of different less frequent carbohydrates by *E. amylovora* besides basic sugars.

## Materials and methods

### Test organism and incubation temperature

*E. amylovora* (strain Ea 1) was isolated from apple in Nyárlőrinc, Hungary (Hevesi, 1996) and preserved by lyophilization. Virulence was confirmed on unripe pear fruits showing typical water-soaked tissue lesions with bacterial exudates and by hypersensitive reaction on tobacco leaves. Standard incubation temperature of each experiment was 26 °C. All media used were adjusted to pH 6.8. Carbohydrates were autoclaved or filter sterilized (0.2µ).

### Effect of different carbohydrate concentrations on bacterial cell multiplication measured photometrically

Solutions of fructose, glucose, sucrose were diluted by Basal medium (Dowson, 1957)  $\{(NH_4)_2H_2PO_4$  1.0 g; KCl 0.2 g;  $MgSO_4 \times H_2O$  0.2 g per liter distilled water} to sugar concentrations of 0.6, 1.2, 2.5, 5.0, 10, 20, 30 g/100 ml in different proportions: 2(F):1(G):1(S); 1(f):2(G):1(s); 1(f):1(g):2(S); (capital letter means double proportion; small letters single proportion of sugars) and separately 3(F); 3(G) and 3(S) as controls. Test tubes were filled with 10 ml solutions inoculated by 100 µl of  $10^8$  cells/ml of 24 h-old cultures of *E. amylovora* (Ea 1). Turbidity of the tubes was followed daily using a spectrophotometer (Cole Parmer 5565-05) at 560 nm for 5 days to demonstrate bacterial cell multiplication.

### Acid production of main sugars (F, G, S separately) on solid medium

Acid production between sugar concentrations of 0.6, 1.2, 2.5, 5 g /100 ml was checked on solid-Basal-medium containing bromthimol purple ( 0.7 ml of a 1.5 % solution in ethanol per litre ). This medium (10 ml) was plated into Petri

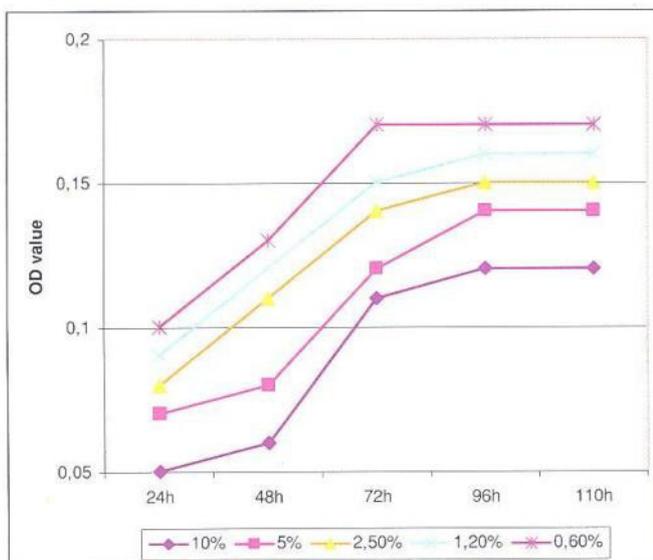


Figure 1. Effect of different sugar concentrations (means of Gfs, Fgs, Sgf) on *E. amylovora* multiplication

dishes which were inoculated in the center by 100 µl bacterial suspension. Color change at the site of inoculation (acid production turns the color from green to yellow) was an indication of utilization of sugars and the resulting bacterial cell multiplication.

### Utilization of various carbohydrates assayed by API 50 CH strip

A 24 h old culture of the strain Ea 1 grown on King-B agar plate was harvested in distilled water and the suspension ( $10^8$ cells/ml) transferred into API medium. As much as 49 capsules containing different carbohydrates were filled and overlaid with mineral oil providing anaerobic conditions for the facultative anaerobic *E. amylovora*. Evaluations were realized daily for five days until the stop of color change in the capsules.

## Results and Discussion

### Effect of sugar (F, G, S) concentration on *E. amylovora* cell multiplication

The three main types of sugars (fructose, glucose, sucrose; F, G, S) that exist in relatively high proportions in the nectar of nearly any plant species are also present with a refraction of up to 30% in apple (Orosz-Kovács *et al.*, 1997, Nagy Tóth *et al.*, 2000) and 4–10% in pear (Benedek *et al.*, 2000, Farkas, 2001). Based on the works of the above authors artificial nectar solutions, i.e. sugar solutions of various composition, similar to natural nectars, were prepared. Using various methods to carry out our investigations, it has been clearly shown that in high concentrations of G, F, S the pathogen *E. amylovora* was not able to multiply. Artificial nectars of high concentrations (30% and 20%) of F+G (the composition, but not the concentration, is a characteristic of pear) and F+G+S (composition and concentration is a characteristic of apple) were unsuitable for bacterial cell multiplication. Bacterial multiplication did not occur at a total sugar concentration of 30%, while at 20% it was negligible and/or not measurable photometrically. Nectar refraction above 30% is supposed to be a restricting factor for development of fire blight (Thomson, 1986).

In a serial dilution of 10–0.6% of Gfs, Fgs and Sfg (capital letter means double proportion; small letters single proportion of sugars) bacterial development was observed. The different proportions of basic sugars (Gfs, Fgs, Sfg) had no significant influence on bacterial cell proliferation (Fig. 1).

### Acid production of *E. amylovora* (Ea 1) in low concentration of F, G, S

Determination of bacterial cell numbers by spectrophotometer (on the basis of turbidity) was not possible in a

**Table 1** Acid production of *E. amylovora* (Ea1) at different concentrations of single sugars

Concentration (%)	Fructose	Glucose	Sucrose
5	+-	++	++
2,5	+-	++	++
1,2	+-	++	++
0,6	+-	++	++
0,3	+	++	++
0,15	+	++	++

Degree of acid production: +- weak  
(Yellow colour) + moderate  
++ strong

suspension below  $10^7$  cfu/ml. The growth of the strain Ea 1 can be demonstrated by an indirect way, too, i.e. by the breakdown of sugars to organic acids (Fig. 2.). Acid production of *E. amylovora* (Ea 1) was evident at different concentrations (0.1–5%) of G, F, S (Table 1).

Our experiment showed that for multiplication *E. amylovora* requires only a small amount of sugars, the optimal concentration was between 0.6–1.2 (Fig. 1). There was a negative correlation between sugar content and cell density. The optimal concentration for cell proliferation seems to be about 1%.

#### Utilization of various carbohydrates by *E. amylovora*

Beside dominant nectar sugar components (F, G, S,) only few data exist in the literature about the effect of other carbohydrates – so called “less frequent sugars” – on *E. amylovora* multiplication. Other sugars as maltose, melibiose and raffinose occurred also in nectar from flowers of some plant species (Percival, 1961) but their effects are unknown.

By the API 50 CH strip method we checked a number of unstudied carbohydrates until the end of reactions (color



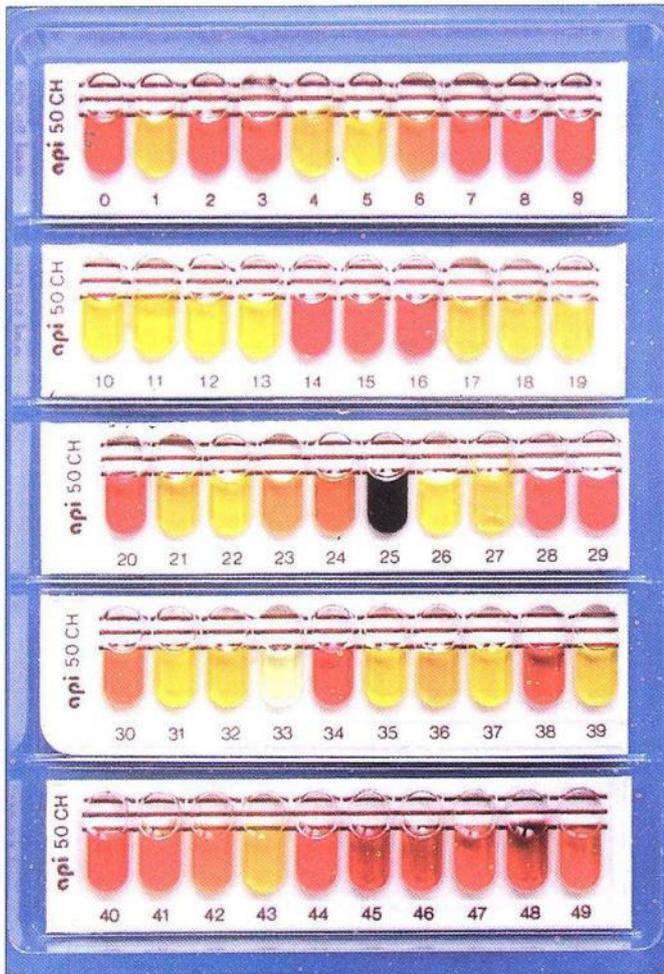
**Figure 2** Acid production of *E. amylovora* (Ea1) on Basal indicator medium 4 days after inoculation (glucose 0.15%)

**Table 2** Carbohydrates utilized by *Erwinia amylovora* strain Ea1 (see also in Figure 3)

Not utilized			
Numbers		Numbers	
0	control		
2	Erythritol	30	Melibiose
3	D Arabinose	34	Melezitose
6	D Xylose	38	Xylitol
7	L Xylose	40	D Turanose
8	Adonitol	41	D Lyxose
9	β-Methyl-D-Xyloside	42	D Tagatose
14	Sorbose	44	L Fucose
15	Rhamnose	45	D Arabitol
16	Dulcitol	46	L Arabitol
20	α-Methyl-D-Mannoside	47	Gluconate
28	Maltose	48	2-Keto-Gluconate
29	Lactose	49	5-Keto-Gluconate
Utilized slowly, weakly (164 h)			
Numbers			
1	Glycerol		
23	Amygdalin		
24	Arbutin		
Utilized slowly, completely (66h)			
Numbers		Numbers	
17	Inositol	35	Raffinose
26	Salicin	36	Starch
27	Cellobiose	37	Glycogen
33	Inulin	39	Gentiobiose
21	α-Methyl-D-Glucoside	43	D Fucose
Utilized quickly, completely (20–26h)			
Numbers		Numbers	
4	L Arabinose	18	Mannitol
5	Ribose	19	Sorbitol
10	Galactose	22	N-Acetyl-Glucosamine
11	Glucose	25	Esculin
12	Fructose	31	Sucrose
13	Mannose	32	Trehalose

changes). We could provide information on a wide scale, concerning utilization of 49 carbohydrates and determine different groups of carbohydrate utilization by the bacterium as follows: Not utilized, Slowly and weakly utilized, Slowly and completely utilized, Quickly and completely utilized carbohydrates (Table 2, Fig. 3). We suppose that carbohydrates that belong to the latter two groups could play an important role in invasion of flowers by *E. amylovora*. Other carbohydrates present in the “Slowly and weakly” utilized group have no role in the invasion of blossoms possibly because dominant sugars prevent their utilization.

It is supposed that there is a relationship between the low sugar concentration of floral nectar and the susceptibility of certain apple and pear cultivars (e.g. ‘Sampion’ and ‘Conference’, respectively). It is true the other way round: flowers in trees of rather tolerant apple and pear cultivars (e.g. ‘Freedom’ and ‘Beurré Bosc’, respectively) secrete nectar of higher refraction value (Orosz-Kovács et al. 2004 and Farkas et al. 2004, in this issue).



**Figure 3** Carbohydrates utilized by *E. amylovora* (Ea1) after 7 days (0-control; 1–49 different carbohydrates; colour changes indicate the decomposition)

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