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Specific Substrates for Isolation and Differentiation of *Azotobacter vinelandii*

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Summary. Resorcinol, ethylene glycol and glutaric acid have been found to be specific substrates for the enrichment of *Azotobacter vinelandii* in the presence of other *Azotobacter* species. The three compounds may also be used for species differentiation in the genus *Azotobacter*. In contrast to other reports strains of *A. chroococcum* and *A. beijerinckii* have been isolated which are able to use L-rhamnose for growth.

Conditions for the production of a yellow pigment and green fluorescence by *A. vinelandii* from non-aromatic substrates on agar-plates have been tested.

The production of water soluble yellow pigments in benzoate containing media cannot be used as a criterium for the presence of *A. vinelandii*. The pigment seems to be α-hydroxy-muconic semialdehyde, the splitting product resulting from meta-cleavage of catechol. It may be formed by all *Azotobacter* species that can metabolize aromatic substrates.

Aerobic enrichment cultures with nitrogen free media and mannitol, benzoate or other carbon compounds usually lead to the development of *Azotobacter chroococcum*. This species seems to be far more abundant in soil than the other members of the genus. It is also often isolated from water or mud. Available enrichment methods for the different species of *Azotobacter* were summarized by H. L. Jensen (1965) and Norris (1959).

For the selective isolation of *A. vinelandii* two methods have been described. Derx (1951) isolated this species using mannitol plus benzoate as carbon sources. According to Derx, suppression of *A. chroococcum* in the enrichment medium is due to benzoate which, in a concentration of 1%, usually inhibits the growth of pure cultures of this species, whereas *A. vinelandii* is not affected. V. Jensen (1961) found that rhamnose can be used for isolation of *A. vinelandii* from soil.

In our enrichment experiments, both methods mentioned did not turn out to be highly selective. Studying the utilization of carbon-compounds by pure cultures, we found that *A. vinelandii* can use several substances for growth which are not suitable for other *Azotobacter* species. The question arose whether these substrates can be used more successfully for the selective isolation of *A. vinelandii*.

Materials and Methods

For enrichment and pure culture studies the following basal medium was used: K_2HPO_4 900 mg; KH_2PO_4 100 mg; $MgSO_4 \times 7\ H_2O$ 100 mg; $CaCl_2 \times 2\ H_2O$ 100 mg; $Na_2MoO_4 \times 2\ H_2O$ 5 mg; $FeSO_4 \times 7\ H_2O$ 12.5 mg; H_2O dest. 1000 ml; pH 7.3. Substrates were sterilized by filtration and were added in a final concentration of 0.2% (w/v) or 0.1% in case of aromatic compounds. In enrichment experiments according to Derx 0.5% mannitol + 1% benzoate, according to V. Jensen 1% L(+)-rhamnose were used. In studies of pigment formation by *A. vinelandii*, substrates were used in a concentration of 0.5%.

30 ml of the above medium in 300 ml Erlenmeyer flasks were inoculated with 5 to 10 g of fresh soil (garden, field, compost) or with 5 ml of water (lake, river). Flasks were incubated at 28° C under static conditions. Four days after inoculation, enrichment cultures were transferred into media of the same composition. If growth occurred, the cultures were streaked on appropriate agar media and finally purified on nitrogen-free glucose agar.

Utilization of substrates was tested by multipoint inoculation with a capillary-action replication apparatus (Hartman and Pattee, 1968) on nitrogen-free agar media. Cells from nitrogen-free glucose agar were used for inoculation.

Pure cultures used in substrate tests were our own isolates and collection strains (*A. beijerinckii* ATCC 19360, *A. vinelandii* ATCC 12837, *A. agilis* ATCC 12838). For identification of the isolated strains the data of Voets and Dedeken, (1966) and Norris and Chapman (1968) were used.

Results

Enrichment of *Azotobacter* According to Derx; Formation of Diffusible, Yellow Pigments from Benzoate by *Azotobacter*

From enrichment cultures of this type we isolated more than 20 *Azotobacter* strains which, on Derx's benzoate-mannitol agar produced yellowish, diffusible pigments. Also, in liquid culture media of the same composition most of these strains formed a yellow colour which with some strains turned to green, brown or black after prolonged incubation.

Further identification of the isolated strains, however, showed that only six could be classified as *A.vinelandii*. The remaining cultures should be grouped with *A.chroococcum* or *A.beijerinckii*.

The formation of water-soluble, yellow pigments is claimed to be specific for *A.vinelandii*, *A.agilis* (Bortels, 1939; Johnstone and Fishbein, 1956) and *A.paspali* (Döbereiner, 1966). For *A.chroococcum* or *A.beijerinckii* no such ability was reported. Further observations showed that in contrast to the first mentioned species the pigment producing strains of *A.chroococcum* and *A.beijerinckii* showed no yellow-green fluorescence. Moreover, with these species a yellow pigment was observed only on benzoate containing media. It is also formed when benzoate grown cells are tested for the type of cleavage of aromatic ring structures with catechol or benzoate as substrate (Pankhurst, 1965; Stanier *et al.*, 1966).

The same results were obtained with other strains of *A. chroococcum*, originally isolated on mannitol or glucose medium. Studies with culture filtrates of benzoate grown *A. chroococcum* and *A. beijerinckii* strains finally showed that the yellow pigment produced is spectrophotochemically identical with α-hydroxymuconic semialdehyde, the product of meta-cleavage of aromatic ring structures. This is in accordance with results recently published by Hardisson *et al.* (1969) who found that benzoate is oxidized by all *Azotobacter* species tested via catechol by meta cleavage. The coloured cleavage product is chemically unrelated to the typical *A. vinelandii* pigments (Bulen and LeComte, 1962; Corbin and Bulen, 1969) which, in general, are formed independently from the substrate used.

Enrichment of *Azotobacter* with Different Substrates

Johnstone (1968), in a review on *Azotobacter* stated that "nutritional methods of distinguishing species has long been one of the best, and after all these years, it is surprising when a common carbohydrate appears to be species—unique".

Systematic studies with a broader spectrum of substrates, however, have not been published. Our studies on utilization of some 100 different substrates by pure cultures of *Azotobacter* species showed that *A. vinelandii* strains can use several compounds for growth in nitrogen-free media which are not suitable for the development of other *Azotobacter* species. In Table 1 the most striking differences are summarized.

Results of enrichment studies with the 4 substrates only used by pure cultures of *A. vinelandii* are listed in Table 2. To be sure that the substrates used were selective for *A. vinelandii*, the soil or water samples were also incubated with non-selective compounds for demonstrating the presence of *A. chroococcum* or *A. beijerinckii*.

With the exception of 2 water samples *Azotobacter* species were present in all samples tested. *p*-Hydroxybenzoate and mannitol sustained growth of only *A. chroococcum* or *A. beijerinckii*. In contrast to the findings of V. Jensen (1961) and our own results with pure cultures (Table 1), we isolated with rhamnose not only *A. vinelandii*, but also cultures of *A. chroococcum* and *A. beijerinckii*. In addition to the substrate spectrum given in Table 1, these strains grow well with rhamnose, but are unable to use the other *A. vinelandii* specific compounds. Resorcinol, ethylene glycol and glutarate were found to be highly selective for the enrichment of *A. vinelandii*. Although the presence of *A. chroococcum* and *A. beijerinckii* in the soil or water samples has been shown by enrichment with non-selective substrates, these species did not develop.

The *A. vinelandii* cultures isolated with the specific compounds showed the same nutritional properties as known from the reference

Table 1. *Substrate utilization by* Azotobacter *species*

Substrate	*A. chroococcum* 30 strains	*A. beijerinckii* 15 strains	*A. vinelandii* 15 strains	*A. agilis* 2 strains
Glucose	30 +	15 +	15 +	2 +
Mannitol	30 +	15 +	15 +	2 —
p-Hydroxybenzoate	30 +	15 +	15 +	2 —
Resorcinol	30 —	15 —	15 +	2 —
Glutarate	30 —	15 —	15 +	2 —
Ethylene glycol	30 —	15 —	15 +	2 —
L-Rhamnose	30 —	15 —	15 +	2 —

+ growth; — no growth.

Table 2. Azotobacter *species isolated with different substrates*

Substrate	Soil or water sample																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
p-Hydroxybenzoate	b	c	c	c	c	b	c	—	c	c	c	b	b	—	c	b	c	c
Mannitol	—	—	c	c	c	b	c	—	c	c	c	—	b	—	c	c	—	c
L-Rhamnose	—	—	—	—	c	v	—	—	—	c	v	b	—	—	v	—	—	—
Resorcin	—	—	v	—	v	v	—	—	—	—	v	—	—	—	—	—	—	—
Ethylene glycol	—	—	v	—	v	v	—	—	—	—	—	—	—	—	v	—	—	—
Glutarate	—	—	v	—	v	v	—	—	—	—	v	v	—	—	v	—	—	—

b = development of *A. beijerinckii* v = development of *A. vinelandii*
c = development of *A. chroococcum* — = no development of *Azotobacter* species.

Table 3. *Production of pigment and fluorescence by* A. vinelandii

Substrate	Fe^{++}	$CaCO_3$	Bacto-Agar Difco		Ionagar No. 2 Oxoid		Agar No. 1 Oxoid	
			P	F	P	F	P	F
Glucose	+	—	1	9	1	15	0	0
	+	+	19	27	10	25	21	26
	—	—	22	24	20	27	14	19
Mannitol	+	—	0	2	0	11	0	0
	+	+	22	24	9	23	11	26
	—	—	19	26	22	28	10	27
Succinate	+	—	12	23	6	27	0	8
	+	+	15	27	9	23	6	18
	—	—	26	27	16	27	21	26

P = pigment formation; F = fluorescence; Number of positive strains are given (total number of strains: 28).

strains (Table 1). The utilization of resorcinol, ethylene glycol and glutaric acid therefore may be used as additional criteria for distinguishing *A. vinelandii* from the other species of the genus.

Production of Yellow and Fluorescent Pigments in *A. vinelandii*

Green fluorescence and production of a diffusible, yellow pigment from non-aromatic substrates is an important aid in species selection and final identification of *Azotobacter* strains. From the work of Bortels (1939) and Johnstone and Fishbein (1956) we know that the formation of a diffusible, yellow pigment and of yellow-green fluorescent substances by *A. vinelandii* is influenced by the available concentrations of iron and molybdenum. With our strains we found that the production of both, pigment and fluorescence was dependent upon the media used. Many strains of *A. vinelandii* would have not been detected if these factors had been used as the only criteria for strain selection.

From Table 3 it is obvious that iron should be omitted from agar media in order to obtain yellow and fluorescent cultures. Alternatively, one may add $CaCO_3$ in a concentration of 0.5%. The effectivity of $CaCO_3$ in adsorbing minor elements from culture media during heat sterilization is well known (Donald *et al.*, 1952).

Whereas the production of the diffusible, yellow pigment varies from experiment to experiment and is influenced by the type of substrate and agar, the presence of fluorescent material in the media without added iron is a more constant property of *A. vinelandii*.

Discussion

Methods for the enrichment of *A. vinelandii* may be of interest for two reasons. In nitrogen-free enrichment cultures the bacterium is obviously outgrown by other *Azotobacter* species. Isolation of *A. vinelandii* without selective enrichment conditions therefore is only at random. Since appropriate selective methods are lacking very little is known about the distribution of *A. vinelandii* in nature. Selective isolation methods may contribute to this problem.

From the results presented it is evident that Derx's method for isolating *A. vinelandii* is not highly selective. Only about 20% of the cultures isolated under these conditions could be classified as *A. vinelandii*. In agreement with Derx (1951), many of our pure cultures of *A. chroococcum* may be inhibited by benzoate concentrations of 1%. We found, however, that it is easy to isolate from soil strains of this species that resist the benzoate concentration used by Derx.

In addition to the production of fluorescent material the formation of a diffusible, yellow pigment is an important aid for species differentia-

tion in the genus *Azotobacter*. It is important, however, to note that the formation of a yellow pigment in benzoate containing culture media cannot be used as a criterion for the presence of *A. vinelandii*. This pigment is presumably identical with α-hydroxy-muconic semialdehyde, a degradation product of benzoate. It may be formed by all *Azotobacter* species that can metabolize aromatic compounds. The typical *A. vinelandii* pigment is produced also from non-aromatic substrates. Its formation depends upon the culture conditions and is not always reproducible. The same holds true for the excretion of fluorescent material. For detecting both pigments the use of agar-media without added iron is recommended during isolation and purification procedures.

Highly selective substrates for isolating *A. vinelandii* include resorcinol, ethylene glycol and glutaric acid. In addition, these compounds are extremely useful in the differentiation of *A. vinelandii* from the other species of the genus, especially since the formation of the yellow pigment and of fluorescent material is not always reproducible.

The failure of about 50 pure cultures of *A. chroococcum* and *A. beijerinckii*, isolated on glucose, mannitol or aromatic compounds, to use L-rhamnose for growth in nitrogen-free media is in agreement with all reports in the literature. The isolation of rhamnose positive strains of these two species after enrichment with this carbohydrate shows, that such variants are present in many soils. However, they are probably present in such low numbers that there is little chance of isolating them under non-selective enrichment conditions.

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