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Isolation of Microorganisms Producing 6-Deoxyhexose-Containing Polysaccharides

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Summary

A screening of 27 collection strains and 135 strains isolated from carbohydrate rich waste materials permitted the selection of 13 strains which produced more 6-deoxyhexose-containing polysaccharides than a reference strain: *Acinetobacter calcoaceticus* BD 413 (ATCC 33304). The most productive strain identified as *Klebsiella* sp. excreted 1.6 g of rhamnose per liter of culture medium after improvement of certain culture conditions. The *Klebsiella* polysaccharide was recovered by precipitation with acetone and hydrolyzed. Rhamnose was separated by chromatography on Dowex 1 x 4 in borate buffer. Our study shows that sewage sludge is a good source of polysaccharide producers and that the use of hydro carbon in selective medium and of a high carbon-nitrogen ratio promotes their development.

Key words: *Klebsiella* sp. - Microbial polysaccharides - Rhamnose - Fucose – Screening

Introduction

Some microbial polysaccharides have a commercial value due to their gel forming or emulsifying properties, while others represent a source of certain important monosaccharides such as the 6-deoxyhexoses (6dh) rhamnose and fucose (Paul et al., 1986). Among the bacterial strains whose polysaccharides' chemical composition is known, about half the strains produce 6dh-containing polysaccharides (Berthelet et al., 1984). Although some of these strains produce up to several g per litre of 6dh-containing polysaccharide, none but one has been studied with the intention of producing 6dh (Voelskow and Schlingmann, 1984). To our knowledge, no microorganism has been reported to excrete free 6dh such as rhamnose or fucose. Screening methods for the selection of polysaccharide producing microorganisms taking advantage of resistance to antibiotics (Rubinovitz et al., 1982) or to phages (Martin, 1973) have been described. However no screening for the isolation of microbial strains producing 6dh-containing polysaccharides from samples taken from the environment has been reported. Soils rich in oily deposits (Wang and Schwartz, 1985), or in hydrocarbon wastes (Guerra Santos, 1985) are favorable for polysaccharide-producing strains. Sewage sludges contain microorganisms producing polysaccharides having 6dh as main components (Kakii et al., 1986; Rideau and Morfaux, 1976). Habitats containing large

amounts of carbohydrate waste materials and small amounts of nitrogenous substrates favour polysaccharide production in many microorganisms (Lawson and Sutherland, 1978).

Our study deals with the screening of microbial strains producing rhamnose- or fucose-containing polysaccharides.

We report the variation in rhamnose yield produced by a *Klebsiella* sp. strain isolated from sewage sludges as a function of growth conditions.

Materials and Methods

Screening

Microbial strains. The following 27 collection strains were purchased from the American Type Culture Collection (ATCC, Rockville, U.S.A.), from the Deutsche Sammlung von Mikroorganismen (DSM, Gottingen, West Germany), from the Institut Pasteur (IP, Paris, France), Central bureau Voor Schimmelcultures (CBS, Barn, Delft, The Netherlands) and from the National Collection of Industrial Bacteria (NCIB, Aberdeen, Great Britain) *Acinetobacter calcoaceticus* BD 413 (ATCC 33304), DSM 586IP 64.7, IP 64.8, IP 6632 and IP 6633, *Alcaligenes* sp. ATCC 31853, *Bacillus polymixa* Ncrn 11429, *Beijerinckia indica* NCrn 8849, *Beijerinckia mobilis* NCrn 9879 and DSM 1720, *Corynebacterium insidiosum* DSM 20157, *Enterobacter sakazakii* ATCC 12868, ATCC 29004 and ATCC 29544, *Pseudomonas elodea* ATCC 31461, *Pseudomonas cepacia* DSM 50180 and DSM 50181, *Rhodotorula glutinis* DSM 70398, *Rhodospiridium toruloides* CBS 14, CBS 350, CBS 5490 and CBS 5475, *Serratia marcescens* ATCC 17999 and *Streptococcus sobrinus* ATCC 33478. *S. sobrinus* strains 6715-T2 and B-13 were gratefully given by Dr. R. Linzer (Buffalo, U.S.A.). The isolation, screening, and maintenance procedure of the unknown strains are shown in Table 1. All culture media are described in Table 2.

The strains were isolated at 30°C after incubation of samples for 3 to 28 days. Attempts to isolate microorganisms at 3°C and 55°C and to isolate yeasts on malt agar (Difco) were abandoned because they led to a small number of poorly growing strains.

Determination of the 6dh. The amounts of 6dh produced by the strains were determined after culture for 2 days in medium K1 or after culture for 3 days on medium BA (Table 2). The collection strains of *Beijerinckia* were cultured on the *Beijerinckia* medium recommended by the DSM, and the *S. sobrinus* strains were cultured on complemented Todd-Hewitt agar (Difco) (Prakobphol and Linzer, 1980). The 6dh produced were assayed by the sulfuric acid-cystein method for methyl pentoses using L-rhamnose as the standard (Dische and Shettles, 1948). The assays were carried out on the 1:10 diluted culture medium K1 or on buffered saline suspensions of colonies grown on BA. Results obtained were compared to the amount

of 6dh produced by *Acinetobacter calcoaceticus* BD 413 (*A. calcoaceticus*) cultured under conditions similar to the tested strains.

Identification of the 6dh produced by thin-layer-chromatography (TLC).

Strains producing more 6dh than *A. calcoaceticus* were cultured in medium B (Table 2) for 3 days. One ml of each culture was mixed with 4 ml of cold acetone and stirred for 30 min at 4°C. The precipitate obtained was dried at 60°C, suspended in one ml of 2N H₂SO₄, hydrolyzed for 2 h at 100°C, neutralised with NaOH and freeze-dried. The dried samples were resuspended in ethanol and qualitative analysis of sugars was performed by TLC (Randerath, 1971). Aliquots of 25 µl were spotted on Kieselgel 60 plates and eluted with butanol: acetone: water (4: 5: 1). The plates were sprayed with a solution containing 10 parts of a 0.2% methanolic dihydroxy-1,3 naphthalene and 1 part of phosphoric acid 85%. Blue to purple spots appeared after heating at 100-110°C for 5-10 min.

Determination of cell-bound and extracellular 6dh.

Strains producing more 6dh than *A. calcoaceticus* were cultured in 100 ml flasks containing 20 ml of medium KI for 5 days at 30°C and at 150 rpm. The media were inoculated with one ml of a bacterial suspension spectrophotometrically standardized at an absorbancy of 0.15 (545 nm). Following incubation, 10 ml culture samples were centrifuged for 20 min at 12000 x g and at 4°C. The wet cell pellets were weighed and resuspended in 10 ml of sodium phosphate buffer 0.1 M at pH 7.0. The amount of cell-associated 6dh (whole cell suspensions), extracellular 6dh (supernatant) and total 6dh (culture medium) were determined by the method of Dische and Shettles (1948).

Culture medium and growth conditions of *Klebsiella* sp (strain 82).

The culture medium K4 (Table 2) for growing *Klebsiella* sp.(strain 82) was derived from experiments described in "Results". The effect of aeration on the growth and the 6dh production was studied in 21 fermentors (Biolafitte) containing 11 of medium K4 at 30°C.

Isolation of the polysaccharides and of the rhamnose produced by *Klebsiella* sp (strain 82).

Klebsiella sp. (strain 82) was cultured for 5 days at 30°C and at 100 rpm in a 2 l flask with 4 baffles containing 250 ml of the medium K4. Following incubation, the culture was autoclaved at 120°C for 20 min prior to centrifugation at 4°C and 12 000 x g or 10 min. The supernatant was precipitated with one volume of cold acetone and the precipitate recovered by centrifugation at 4°C and 12 000 x g for 10 min.

The polysaccharides were hydrolyzed with 2N H₂SO₄ at 100°C for 6 h, neutralized with NaOH and freeze-dried. Samples from the dried polysaccharides were resuspended in the equilibration buffer of the column used to separate the individual sugars from the hydrolysate. A 40 x 1.5 cm column filled with the resin Dowex 1 x 4 practical grade (OH-) equilibrated with borate buffer (0.11 M sodium tetraborate and 0.17 M boric acid, pH 8.8)

was used according to the method of Floridi (1971). Prior to chromatography, the resin was washed with 2N NaOH followed by water, then with 0.5 M boric acid followed by water and finally with the equilibration buffer. A sample of one ml was applied to the column eluted at 25°C with the equilibration buffer at a rate of 1 ml min⁻¹. Four ml fractions were collected and assayed for their hexose content by the anthrone method.

Components of the different peaks obtained were identified by TLC by comparison with sugar references.

Results

Screening

Selection of strains producing more 6dh than A. calcoaceticus.

Thirteen strains listed at the bottom of Table 3 were found to produce more 6dh than *A. calcoaceticus* when cultured in 20 ml of medium K1. Most of these strains were isolated from sewage sludge. No soil isolate produced 6dh while only two out of 27 collection strains described as containing 6dh were found positive in this respect.

Identification of 6dh produced by TLC. Among the strains producing more 6dh than *A. calcoaceticus*, strains producing a rhamnose-containing polysaccharide were: *Beijerinckia mobilis* NCIB 9879 and DSM 1720, strains 82 (identified as *Klebsiella* sp.), 92, 93, 97, 105. Strain 134 produced a fucose-containing polysaccharide. Strain 64 produced rhamnose- and fucose-containing polysaccharide.

6dh produced by strains 110, 141, 143, 144 could not be determined clearly by TLC.

Determination of total, cell-bound and extracellular 6dh.

Among the 13 strains selected as producing more 6dh than *A. calcoaceticus*, four were discarded (no 97, 110, 143 and *Beijerinckia mobilis* DSM 1720) since they did not reach the same level of 6dh production as *A. calcoaceticus*, when subcultured. Nine strains produced more 6dh than *A. calcoaceticus* (Table 4). The strain 82 (*Klebsiella* sp.) was selected for further investigation. The medium K1 promoted a better growth of the strains isolated in medium H (strains 93, 134, 141, 144) than the medium H itself. Nevertheless growth of these strains in medium K1 was low. For unexplained reasons, 6dh assayed in the supernatant and in the pellet suspension were very low for strains 134, 141, 144.

Parameters of components influencing the production of rhamnose by Klebsiella sp. (strain 82)

Mannitol or sorbitol and L-phenylalanine promoted the production of the rhamnose-containing polysaccharide (Table 5). Two phosphate concentrations (media K2 and K3) were

tested. The lower concentration promoted a higher production of rhamnose. The different parameters or components listed in Table 6 were found to enhance the production of rhamnose by *Klebsiella* sp. (strain 82).

Neither the stationary phase of growth nor the maximum production of the polysaccharide from *Klebsiella* sp. (strain 82) were reached when grown in an aerated fermentor after 5 incubation days (Fig. 1). In a non-aerated fermentor both maximum growth and polysaccharide production were reached after the third incubation day. Although growth was much more important in the non-aerated fermentor, rhamnose production was much lower than in the aerated fermentor.

Isolation of the polysaccharide and of the rhamnose produced by Klebsiella sp. (strain 82)

The culture supernatant (medium K4) used for the isolation of the polysaccharide of *Klebsiella* sp. (strain 82) contained 1.6 g/l of rhamnose. 90% of the polysaccharide was precipitated by one volume of acetone or two volumes of ethanol. The polysaccharide was not precipitated with cetylpyridinium chloride concentration up to 17% (w/v), and it was not adsorbed to chromatographic supports such as CM - or DEAE-Sephadex (Pharmacia). These two last results suggest that the polysaccharide is neutral. Following precipitation of the polysaccharide by acetone, hydrolysis, neutralization and freeze-drying, the monosaccharides were separated as borate-sugar complexes by chromatography on Dowex 1 X 4. The sugar components of the polysaccharide of *Klebsiella* sp. (strain 82) were identified as rhamnose and glucose by TLC. The presence of glucose was also confirmed by the enzymatic analysis of the hydrolysate using the YSI glucose analyser (YSI model 23 A glucose analyser).

Discussion

Rhamnose and fucose are 6-deoxyhexoses (6dh) which can be used as starting material by the flavouring industry.

The purpose of our work was to find screening and culture conditions leading to the isolation of microorganisms producing 6dh-containing polysaccharides. We did not isolate any microorganisms excreting free rhamnose or free fucose in the culture medium. We confirmed the presence of microorganisms producing 6dh-containing polysaccharides in sewage sludges (*Kakii et al.*, 1986; *Rideau and Morfaux*, 1976) since nine strains, out of thirteen positive strains, were selected from sludge samples, in particular strain 82 (*Klebsiella* sl.) producing much more 6dh than the other isolates. Hydrocarbons effectively favour the development of polysaccharide-producing strains (*Guerra Santos*, 1985) since the most efficient isolation culture medium was medium H containing hexadecane.

The assay of the 6dh produced using the method of *Dische* and *Shettles* (1948) was efficient since it permitted to detect the 6dh-producing strains and to quantify the production of 6dh in defined culture media. The choice of the culture media used for the determination of the 6dh produced was based on their ability to permit sufficient growth of all strains while not interfering with the 6dh assay. Moreover liquid media were preferred to solid ones, since it was impossible to assay the 6dh of a microbial colony taken with its agar support as agar interfered with the assay. The colonies of a strain grown on a solid medium had thus to be suspended in saline before the 6dh assay. If some polysaccharides had diffused in agar, they consequently escaped the assay. 6dh production of strains selected in medium H and collection strains was nevertheless estimated after culture in medium B, which interferes with the 6dh assay, as these strains had a sufficient growth only in medium B.

The present screening permitted the selection of 13 strains producing more 6dh than *A. calcoaceticus*. It is known that a number of cultural conditions such as the concentration and source of carbon, nitrogen, phosphorous, aeration, temperature and pH, determine the production of polysaccharides (*Sutherland*, 1972; 1977; *Troy II*, 1979). Furthermore, the sources of carbon are likely to modify the sugar composition of polysaccharides (*Bryan et al.*, 1986; *Kaplan and Rosenberg*, 1982). The improvement of the composition of the *Klebsiella* sp. (strain 82) culture medium led to medium K4 having a carbon/nitrogen ratio close to 70, and resulting in a 6dh production of 1.6 g.l⁻¹.

The number of techniques available to achieve recovery and purification of microbial polysaccharides is large and the methods used depend on the strain studied (*Aspinall*, 1982). The recovery of the polysaccharide from *Klebsiella* sp. (strain 82) 45% of which appeared to be cell-associated, was performed using simple methods such as autoclaving and centrifugation. Following solvent precipitation and acid hydrolysis, the separation of the borate-sugar complexes on Dowex 1 x 4 was successful in that rhamnose was clearly separated.

Optimization of the culture conditions and of the recovery of the 6dh-containing polysaccharide and the recourse to genetic modification could further enhance the yield of 6dh obtained from *Klebsiella* sp. (strain 82).

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Table 1. Isolation, screening and maintenance procedure

Source of microorganisms	Abbreviations	Isolation medium ^a	Screening medium ^a	Maintenance medium ^a
Collection strains	ATCC, DSM, IP, NCIP, CBS	None	BA or special media	BA or special media
Isolates from:				
Humic soil	S	H, HA	BA	} BA
Dung	D			
Oily deposits	O	H	BA	
		RA, BA	K1	
Sewage sludge ^a (Clarification)	Cla S	Strains isolated on medium H were screened on medium BA and strains isolated on media RA and BA were screened on medium K1.		
Sewage sludge (Stabilization)	Sta S			
Sewage sludge (Recycling)	RS			
Dried sludge	DS			

^a The final pH of all media was 7.0 ± 0.1 after sterilization.

^b The sewage sludge samples were taken from the wastewater treatment plant of Ramonville St-Agne (Toulouse, France).

Table 2. Composition of the culture media in g.l⁻¹

Component	media						
	H	R	B	K1	K2	K3	K4
K ₂ HPO ₄	3.0	3.0	–	3.0	9.0	22.0	9.0
KH ₂ PO ₄	1.5	1.5	–	1.5	3.0	7.0	3.0
(NH ₄) ₂ SO ₄	1.0	1.0	–	1.0	1.0	1.0	–
MgSO ₄	5.5	5.5	–	5.5	5.5	5.5	0.2
KCl	0.3	0.3	–	–	–	–	–
NaNO ₃	1.5	1.5	–	–	–	–	–
CaCl ₂	0.03	0.03	–	–	–	–	–
FeCl ₃	0.002	0.002	–	–	–	–	–
MnSO ₄	0.002	0.002	–	0.002	.002	.002	–
H ₃ BO ₃	0.0004	0.0004	–	0.0004	.0004	.0004	–
CoCl ₂	0.0002	0.0002	–	0.0002	.0002	.0002	–
CuSO ₄	0.0002	0.0002	–	0.0002	.0002	.0002	–
Yeast extract	0.02	0.02	–	0.02	.02	.02	.02
Hexadecane	25.0	–	–	–	–	–	–
Rhamnose	–	10.0	–	–	–	–	–
Brain heart infusion	–	–	37	–	–	–	–
Glucose	–	–	40.0	4.0	–	–	–
Mannitol or Sorbitol	–	–	–	–	10	10	10
Phenylalanine	–	–	–	–	1	1	1
Tween 80	–	–	–	–	1	1	1

^a When these media were used as solid media, agar 2% (w/v) was added. Media with agar were named HA, RA, BA.

Table 3. Number of strains producing more 6dh than *A. calcoaceticus* / Number of strains isolated

Isolation medium (incubation time)	Collections ^b	Source of microorganisms ^a						
		S	D	O	Cla S	Sta S	RS	DS
HA, (3 days)	No	0/9	0/12	–	–	–	–	–
H, (0–1 week)	isolation	0/7	0/7	0/4	0/3	–	0/4	0/2
H, (1–2 weeks)	necessary	0/8	0/4	–	–	–	–	–
H, (2–4 weeks)		0/4	1/4	1/3	1/4	2/3	0/3	0/2
RA, (3 days)		–	–	0/5	1/6	0/2	1/5	1/5
BA, (3 days)		–	–	0/7	0/3	1/11	1/4	1/4
Total of positive strains ^c	2/27	0/28	1/27	1/19	2/16	3/16	2/16	2/13 13/162
Strains number	<i>B. mobilis</i> NCIB 9879 and DSM 1720	–	110	144	97 134	93 141 143	82 105	64 92

^a See Table 1 for the meaning of the abbreviations.

^b Collection strains: ATCC, DSM, NCIB, IP, CBS, Private Collection, see “Materials and Methods”.

^c A positive strain produced more 6dh than the reference strain *A. calcoaceticus*.

Table 4. Strains producing more 6dh than *A. calcoaceticus* and excreting more than 50% of the 6dh-containing polysaccharide

Strain	A	B
<i>A. calcoaceticus</i>	80	88
<i>B. mobilis</i> NCIB 9879	95	71
64	96	83
82 (<i>Klebsiella</i> sp.)	842	55
92	102	87
93	104	65
105	265	54
134	128	–
141	158	–
144	190	–

A = 6dh concentration in the culture medium ($\text{mg} \cdot \text{l}^{-1}$).

B = % of extracellular polysaccharide.

Table 5. Effect of carbon and nitrogen source on the production of the rhamnose-containing polysaccharide by *Klebsiella* sp. (strain 82)

Carbon source ^a	Rhamnose concentration (mg · l ⁻¹)	Nitrogen source ^b	Rhamnose concentration (mg · l ⁻¹)
Calcium acetate	1	Sodium nitrate	76
N-hexadecane	1	Glycine	84
Ethanol	37	Sodium nitrite	131
Corn steep	119	Cysteine	216
Sodium acetate	140	Tryptophane	253
Citric acid	147	Yeast extract	377
Lactic acid	227	Ammonium sulfate	840
Glycerol	694	Threonine	910
Glucose	840	L-phenylalanine	1107
Mannitol	870		
Sorbitol	880		

^a nitrogen source: (NH₄)₂ SO₄ (1 g · l⁻¹)

^b carbon source: mannitol (4 g · l⁻¹)

Table 6. Parameters or components influencing the production of the rhamnose-containing polysaccharide by *Klebsiella* sp. (strain 82)

Parameter or component	Range assayed	Optimal value	Total rhamnose (mg · l ⁻¹)
medium K1			840
medium K2			1107
Initial pH of medium K2	5-9	7	1336
Mannitol (g · l ⁻¹)	5-80	10	1484
L-phenylalanine (g · l ⁻¹)	0.062-4	1	1486
Tween 80 (g · l ⁻¹)	0-100	1	1600

Fig. 1. Growth and production of the rhamnose-containing polysaccharide by *Klebsiella* sp. (strain 82) in aerated (0) and non aerated (*) 2 l fermentor. (... = absorbance at 545 nm; - = total rhamnose)

