Microbial Transesterification of Sugar-Corynomycolates

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Growing cells of *Arthrobacter sp.* DSM 2567 and of the bacterium M9b were able to synthesize cell-bound glycolipids. The carbon source determined the type of sugar moiety, whereas the α-branched-β-hydroxy-fatty acid remained constant. The incubation of resting cells of both bacteria in a simple buffer medium with various carbohydrates, including oligosaccharides and derivatives, led to the corresponding carbohydrate-corynomycolates. Predominantly an enzymatic transesterification step seems to be responsible for the formation of new glycolipids; this was established by analytical data and ^{14}C-labeling studies. Concerning the substrate specifity, the transesterification reaction is characterized by a regioselective manner: (i) only the primary hydroxy-groups at C-6 (mono- and disaccharide) or C-6′ (disaccharide) were acylated; (ii) 1-β- or 4-β-substituents (including hydroxy groups) prevented the esterification; (iii) as for disaccharides with only one nonreducing moiety, the C-6- and C-6′-positions were esterified one after another. In the presence of two nonreducing molecular ends with two possible C-6-esterification positions, both positions were substituted simultaneously.

With regard to the studies on localization of the esterase after cell disruption, we found the enzymatic activity bound to the cell debris. In transesterification reactions comparable to resting whole cell experiments, the cell particles also were able to produce disaccharide-corynomycolates.

The enzymatic synthesis of triglycerides has been studied for a long time, but the biochemical synthesis of carbohydrate esters from sugar and fatty acids has been reported only recent. In 1984 Seino et al. succeeded in the esterification of sucrose, glucose, fructose and sorbitol with fatty acids (stearic, oleic and linoleic) by using the lipase of *Candida cylindracea* in a buffer solution (1-3). Anhydrous systems like pyridine were used by Therisod and Klibanov (4), who prepared glucose lipids by a lipase-catalyzed (dried porcine pancreatic lipase) transesterification with trichloroethyl-esters of carboxylic acids like laurate, resulting in the incorporation of the fatty acid in the C-6-position of glucose.

In 1984, our studies with resting cells of *Arthrobacter sp.* DSM 2567 led to the directed synthesis of different glycolipids: After growth on glucose and formation of glucose-corynomycolates, the harvested cells transferred the α-branched-β-hydroxy fatty acids from the glucose to other mono-, di- or trisaccharides (mannose, cellobiose or maltotriose) fed for bioconversion in a simple phosphate buffer (5). Using resting cells we continued the studies of Brennan et al. (6) and Suzuki et al. (7, 8), who produced glucose-, fructose- and sucrosecorynomycolates by *Corynebacterium diphteriae* and *Arthrobacter paraffineus,* respectively, during growth on the corresponding carbohydrates.

In high salinity solution, our carbohydrate-corynomycolates showed good surfactant properties. Cellobiose- and maltose-monocorynomycolates reduced the interfacial tension from 42 to one mN/m at critical micelle concentrations below 20 mg/l in the two-phase system n-hexadecane/salt water.

In addition to these experiments, we have tried to optimize the conditions for growth and resting cell experiments, to isolate the esterase responsible for the esterification of the new carbohydrate with the fatty acids, and to study the substrate specificity including steric effects.

EXPERIMENTAL PROCEDURES

Materials. Arthrobacter sp. DSM 2567 and the bacterium M9b were used as biocatalysts. The carbohydrates were purchased from Merck (Darmstadt, Federal Republic of Germany), Sigma (Deisenhofen, FRG), Serva (Heidelberg, FRG) and Fluka (Neu Ulm, FRG). p-Nitro-phenyl-α-D-maltoside was obtained from Boehringer (Mannheim, FRG).

Growth conditions. Both microorganisms were maintained on YM agar slants.

Concerning *Arthrobacter sp.* a seed medium was prepared by inoculating one platinum loop of the strain into a 500-ml erlenmeyer flask with 100 ml of a sterilized culture medium comprising 2.5 g/l of $(NH_4)_2SO_4$, 1 g/l of KH_2PO_4, 2.5 g/l of Na_2HPO_4, 0.5 g/l of $MgSO_4 * 7H_2O$, 0.22 g/l of $CaCl_2 * 2H_2O$, 0.13 g/l of $FeCl_3 * 6H_2O$, 0.09 g/l of $MnSO_4 * 1H_2O$, 0.005 g/l of $ZnSO_4 * 7H_2O$, 1.2 g/l of citric acid * $1H_2O$, 1 g/l of yeast extract, and 15 g/l of glucose (pH adjusted to 6.8 with 2M NaOH).

After incubation for 24 hr at 100 rpm (Shaker KF4, Infors, München, FRG) and 30°C, 25 ml of this culture were inoculated into 500 ml of the same medium in a two-l shake flask. This cultivation was performed under the same conditions for 96 hr.

The composition of the medium for growth of the bacterium M9b was similar to the medium mentioned above. Exceptions: $(NH_4)_2SO_4$, four g/l instead of 2.5 g/l; no yeast extract; 20 g/l sucrose instead of glucose; pH 6.5 instead of 6.8 at the beginning. Cell cultivation was performed at 30°C as follows:

Preculture I: 100 ml medium in a 500-ml Erlenmeyer flask, 24 hr incubation.

Preculture II: 4 * 500 ml medium in two-l shake flasks (inoculated by 20 ml culture broth/500 ml fresh medium), 24 hr.

Main culture: 20-l bioreactor with intensor system (Giovanola Frères, Monthey, Switzerland), inoculation of 18 l fresh medium by two l culture broth, stirrer speed 1500 rpm, aeration rate: 0.5 v/v/m, 72 hr.

Resting cell conditions. After growing, the cells were harvested by centrifugation at 4°C and 5000 rpm (model J-6b, Beckman, München, FRG) for 10 min and washed twice with 0.08 M phosphate buffer, pH 5.3.

Depending on the conditions of the following studies, different portions of the wet biomass were transferred into 0.08 M phosphate buffer, pH 6.3, mostly supplemented with 30 g/l of a carbohydrate. The reaction mixtures were incubated at 100 rpm and 30°C up to 24 hr.

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Growth and resting cell conditions in the presence of (U-^{14}C)-labeled sucrose. The bacterium M9b was grown on the nutrient mentioned above, supplemented with 3.7 MBq (= 100 μCi) (U-^{14}C)-labeled sucrose/l. After four days at 30°C and 100 rpm the biomass was separated by centrifugation, washed twice with 0.08 M phosphate buffer, pH 6.5, and incubated with 10 g/l mannose in 50 ml of the same buffer. The dry weight of the biomass amounted to about 20 g/l. The conversion was examined by the biomass analysis of 5-ml samples, taken at 0, 24 and 48 hr.

After centrifugation, the residual cell mass was extracted with three ml ethyl acetate; 100 μl of crude organic extract were analyzed by TLC (conditions later). For quantitative measurement of (U-^{14}C)-labeled compounds the TLC- Multitrace-master LB 512 (Berthold, Wildbad, FRG) was used.

Cell disruption. Sixty g wet cell mass were suspended in 0.08 M phosphate buffer, pH 6.3, to a volume of 140 ml. After addition of glass beads (d = 0.25-0.5 mm) to a total volume of 350 ml the cells were disrupted discontinuously by a glass bead mill Dispermat F1 (VMA Getzman GmbH, FRG) at 3000 rpm and water cooling for 30 to 60 min. Then the beads were separated from the suspension by pressure filtration through a teflon-gauze filter. The suspension containing cell debris was centrifugated at 18000 rpm (Zeta 20, Heraeus Christ, Osterode, FRG) and 4°C for 20 min. For the following transesterification reactions of the glucose-corynomycolate, the residual cell particles corresponding to the original cell mass before disruption were used.

Estimation of biomass, $(NH_4)_2SO_4$ and carbohydrate. Ten ml of the whole culture broth were centrifugated for 20 min at 13500 rpm (Labofuge 15000, Heraeus Christ, Osterode, FRG), Ammonium and glucose concentrations in the supernatant were determined by the method of Fawcett (9) and by a glucose analyzer (Beckman, München, FRG) using the glucose oxidase reaction (Boehringer, Mannheim, FRG), respectively. Sucrose was determined by the anthrone method (10). The residual cell mass was dried at 105°C for 48 hr before weight measurement.

Estimation of glycolipid contents. Depending on the different studies, samples of comparable quantities were taken from whole broth or resting cell reactions. After centrifugation at 5000 rpm for 15 min, the biomass was extracted first with CH_2Cl_2, then CH_2Cl_2/CH_3OH (2/1, v/v) and last, n-butanol. The solutions were combined, membrane filtrated (d = 0.2 μm, type SM 16249, Sartorius, Göttingen, FRG), and the solvent evaporated. The extracted products were subjected to quantitative TLC or HPLC. Analytical conditions were as follows:

TLC 1: Stationary phase, TLC-plates (NR.5554, Merck, Darmstadt, FRG); developing system, $CHCl_3/CH_3OH/H_2O$ = 60/15/2 (v/v/v); detecting reagent, anisaldehyde/sulfuric acid/acetic acid = 1/2/100 (v/v/v), 150°C; yellow or green spots in case of glycolipids; quantitative measurement, coupling of above color reactions with light absorption at 590 nm using a High Speed TLC-Scanner, Model CS-920 (Shimadzu, Düsseldorf, FRG).

TLC 2: Stationary phase, Chromarods S II (SES GmbH, Niederolm, FRG); developing system, $CHCl_3/CH_3OH$ = 90/10 (v/v); quantitative measurement, coupling of TLC with FID using an Iatroscan TH 10 (SES GmbH, Niederolm, FRG).

HPLC: Pump, two 112 solvent delivery module (Beckman, München); injector, organizer 340, 20 μl sample-loop (Beckman); detection, UV-detector SPD-2A (Shimadzu, Düsseldorf, FRG); integration: Sigma 10B chromatography data station (Perkin Elmer, Überlingen, FRG); column, silicagel, 5 μm, 4.6 * 250 mm (Serva, Heidelberg, FRG); flow rate, 1.8 ml/min; developing system, n-hexane/tetrahydrofuran (100/0 - 80/20, v:v). Purified glycolipids served as standards.

Isolation and identification of the glycolipids. The carbohydrate-corynomycolates of the crude organic extract were purified by silica gel phases using column or thick layer chromatography.

Identification of the molecular structures: see (5).

RESULTS AND DISCUSSION

Growth and de novo glycolipid formation. As to *Arthrobacter sp.* DSM 2567, we first tested various carbon sources for growth and de novo glycolipid formation. Besides hydrophilic carbohydrates we used lipophilic substances like n-alkanes and triglycerols. Table 1 summarizes the results concerning the biomass production by shake flask experiments. Because the small quantities of yeast extract and citric acid in the nutrient led to 0.5 g/l biomass during studies without additional carbon source, this value is subtracted in all cases. Significant growth was observed after incubation with glucose, fructose, mannose, sucrose, triolein and n-alkanes. During growth on the latter sources, the cells were located within the organic phase or at the interphase between phases. Concerning the simultaneous biosynthesis of

TABLE 1

Biomass Production of *Arthrobacter sp.* DSM 2567 on Various Carbon Sources[a]

Carbon source	Biomass (g/l)[b]	Glycolipid (mg/g biomass)
Glucose	4.3	100
Mannose	2.5	50
Fructose	3.7	0
Palatinitol	0.0	0
Sucrose	4.0	90
Maltose	0.9	20
Lactose	0.2	0
Lactulose	0.2	0
Palatinose	0.0	0
Cellobiose	0.0	0
Melibiose	0.0	0
Melezitose	0.4	0
Amygdalin	0.4	0
Triolein	3.8	0
n-C14,15-alkanes	4.0	20[c]
Hexadecane	4.1	20[c]

[a]Conditions: 100 ml medium; 15 g/l carbon source; T, 30°C; t, 72 hr.
[b]Dry weight.
[c]Trehalose lipids.

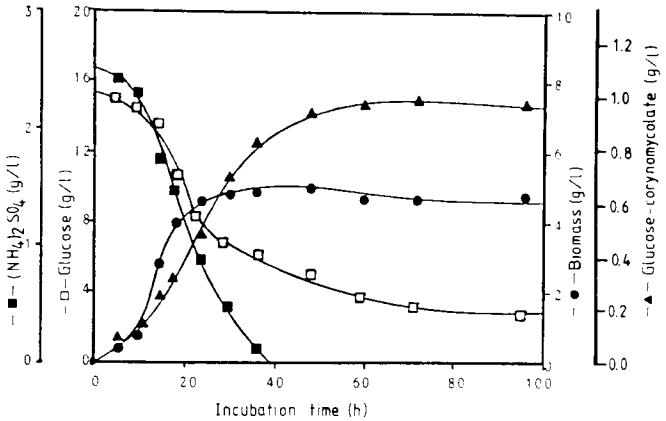

FIG. 1. Cultivation of *Arthrobacter sp.* in two-l shake flasks on glucose as carbon source.

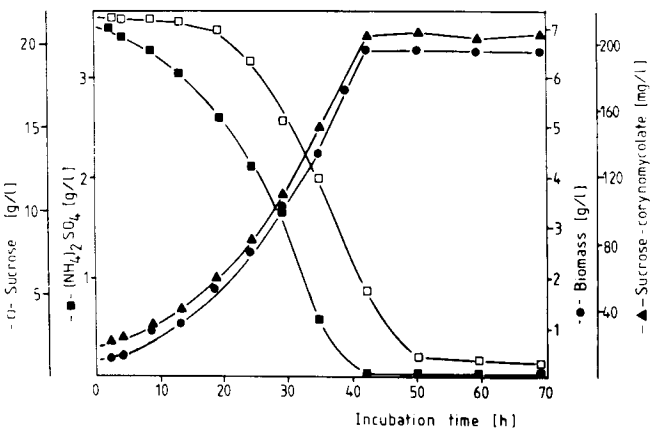

FIG. 2. Cultivation of the bacterium M9b in a 20-l bioreactor on sucrose as carbon source.

glycolipids by carbohydrates, only glucose, mannose, sucrose and maltose (with reservation) led to the corresponding cell-associated sugar-corynomycolates. In the case of the n-alkanes the known trehalose-6-mono and 6,6′-dicorynomycolates (11, 12) and the trehalose-2,2′,3,4-tetraester (13, 14) were obtained. With triolein no glycolipid was produced. As our preliminary studies with glucose were successful with regard to growth and formation of sugar-corynomycolate, we investigated this process in detail. Figure 1 shows a typical cultivation using two-l shake flasks with 500 ml medium. After a lag phase of four hr the biomass increased to at least five g/l (40 hr), corresponding to a yield coefficient y x/s = 0.42 (g biomass/g substrate), related to utilized glucose. At the same time the ammonium ion concentration approached zero. The amount of glucose-corynomycolate increased almost parallel to growth up to 1.0 g/l (= 200 mg/g biomass).

The preliminary investigations on growth and glycolipid formation with the bacterium M9b using the same conditions were qualitatively similar to the above results. As sucrose promoted the growth very well, the production of the more hydrophilic sucrose-monocorynomycolate with this strain was carried out in a 20-l bioreactor with intensor system. The results are presented in Figure 2. Whereas the concentrations of nitrogen and carbon sources decreased almost to zero after 40–50 hr, the amounts of biomass and the polar sucrose lipid increased to their maximum values during the same time. The final yield of sucrose-corynomycolate was 205 mg/l, corresponding to 30 mg/g biomass.

Formation of carbohydrate-corynomycolates by resting cells of Arthrobacter sp. The results of initial studies were as follows:

(i) To get new glycolipids by the esterase activity of the bacterium, resting cells should be used.
(ii) Cells containing glucose-corynomycolate (origin: growth on glucose) were best suited for transesterification reactions.
(iii) The estimation of the optimum timing of cell harvesting for high esterase activity showed that cells separated from the culture broth after 60 hr possessed the highest glycolipid productivity.
(iv) Optimum values for resting cells were 0.08 M phosphate buffer, pH 5.8–6.3, T = 30°C, 30 g/l carbohydrate.

Using increasing concentrations from 1 g/l to 20 g/l (dry weight) of cells, associated with glucose-corynomycolate, and mannose as acceptor within the same reaction volume, the amount of mannose-corynomycolate increased simultaneously. But the ratio of 150 mg mannose lipid/g biomass stayed constant. In the following about 70 g/l wet biomass (corresponding to 12 g/l dry weight) were used in resting cell reactions. We tested various carbohydrates and carbohydrate derivatives for esterification by glucose-corynomycolate associated cells. Table 2 summarizes the results about the yield of glycolipids and the R_F-values (TLC) of mono- or dicorynomycolates. ^1H- and ^{13}C-NMR spectroscopy and elemental analysis accompanied by mass spectroscopic studies on the fatty acids and application of HPLC to the sugars after alkaline hydrolysis show that the lipophilic moieties consisted of a mixture of homologous α-branched-β-hydroxy fatty acids with chain lengths between 32 and 43 carbon atoms including some unsaturation. In general the sugars were esterified in position 6 and 6′ (di- or trisaccharide). The 4-hydroxy groups in the β-position (e.g., galactose) and 1-β-substituents prevented enzymatic esterification, which could be an indication of steric hindrance.

Structures of the cellobiose-6-monocorynomycolates are represented in Figure 3. It is important to mention that initial experiments with growing cultures on cellobiose were negative because of the lack of β-1,4-glucosidase. Therefore, this glycolipid supports our supposition that a transesterification step is involved in the formation of carbohydrate-corynomycolates. The relatively high yields of sucrose-, maltose- and the melezitose-lipids, compared to the original glucose-lipid concentration at the cells, could be explained as follows: (i) The molecular weights of the sugar mentioned above are higher than that of glucose. (ii) The carbohydrates added in excess concentrations may be metabolized by the bacterium under limiting conditions, possibly followed by new biosynthesis of fatty acids.

(U-^{14}C)-labeling studies on the formation of carbohydrate-corynomycolates with the bacterium M9b. To show that the corynomycolic acids of a definite glycolipid (produced during growth on a certain carbohydrate) are

TABLE 2

Synopsis of All Carbohydrate-Corynomycolates Produced by Resting Cell of *Arthrobacter sp.* DSM 2567 (after growth on glucose)[a]

Carbohydrate	Glycolipid (mg/g biomass)	R_F-value (TLC) Mono-	Diester
Fructose	100	0.62	
Mannose	150	0.59	
Galactose	-[b]		
Sucrose	300	0.36	0.83
Palatinose	180	0.31	0.72
Lactulose	-[b]		
Lactose	-[b]		
Maltose	300	0.22	0.60
Isomaltose	120	0.18	
Gentiobiose	100	0.17	
Cellobiose	80	0.20	
Melibiose	-[b]		
Melezitose	220	0.15	0.68
Raffinose	-[b]		
Maltotriose	20	0.09	
Stachyose	-[b]		
Maltitol	160		0.74
Palatinitol	170		0.75
p-Nitrophenyl-1-β -D-glucose	-[b]		
-D-mannoside	-[b]		
p-Nitrophenyl-s1-α -D-glucoside	100	0.73	
-D-mannoside	80	0.74	
-D-maltoside	120	0.48	
-D-maltotrioside	40	0.23	
-D-maltotetraoside	traces	0.13	
Amygdalin	110	0.40	
Sophoroselipid, acidic	60	0.42	

[a]Conditions: 100 ml, 0.08 M phosphate buffer, pH 6.3; 12 g/l biomass (dry weight), concentration of glucose-corynomycolate: 200 mg/g biomass, R_F-value: 0.56.

[b]Corynomycolates of these sugars couldn't be observed.

transferred to a second sugar molecule in resting cell experiments, we cultivated the bacterium M9b on (U-^{14}C)-labeled sucrose. After harvesting, the cells containing sucrosemonocorynomycolate were incubated in the presence of excess mannose for at least 48 hr. Because the R_F-value of mannose corynomycolate (0.59) was higher than that of sucrose monoester (0.36), the

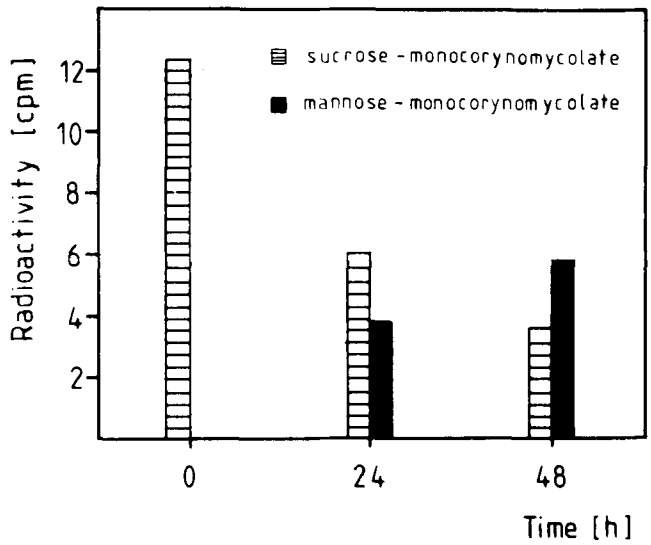

FIG. 4. Transesterification of sucrose-6-corynomycolate to mannose-6-corynomycolate by resting cells of bacterium M9b (after growth on sucrose). Analysis by (U-^{14}C)-labeling experiments on the organic extracts.

following ^{14}C- measurements by a TLC scanner were facilitated. Figure 4 presents the result: The radioactivity of the original glycolipid at t = 0 hr has been changed with time by increasing tendency to the new one; the total amount decreased corresponding to the replacement of labeled sucrose by unlabeled mannose. Additionally, only traces of free ^{14}C-labeled corynomycolic acids were observed in the crude organic extract of cells after growth.

The results of these studies surely are valid for *Arthrobacter sp.* DSM 2467 and seem to prove the concept of a transesterification step.

Comparison: Resting cells/cell particles after cell disruption. To look for (i) the esterase; (ii) extracellular protein, and (iii) the highest values of some key enzyme activities like isocitric acid dehydrogenase or glucose-6-phosphate dehydrogenase in the supernatant of a cell disruption suspension, we tested an ultrasonic disruptor and a glass bead mill. By using the latter device, we succeeded in getting the highest values of protein and key enzyme activities mentioned above (15) within the supernatant. However, our target enzyme, the esterase, was not soluble but located in the cell debris. Therefore,

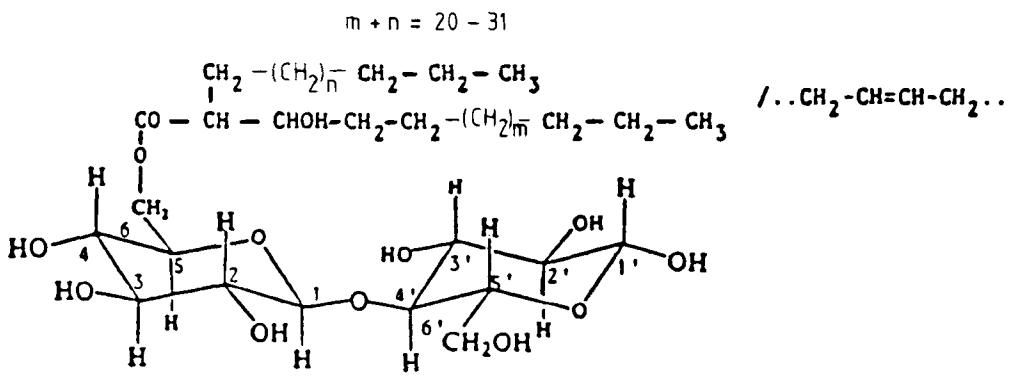

FIG. 3. Structure of cellobiose-6-monocorynomycolate.

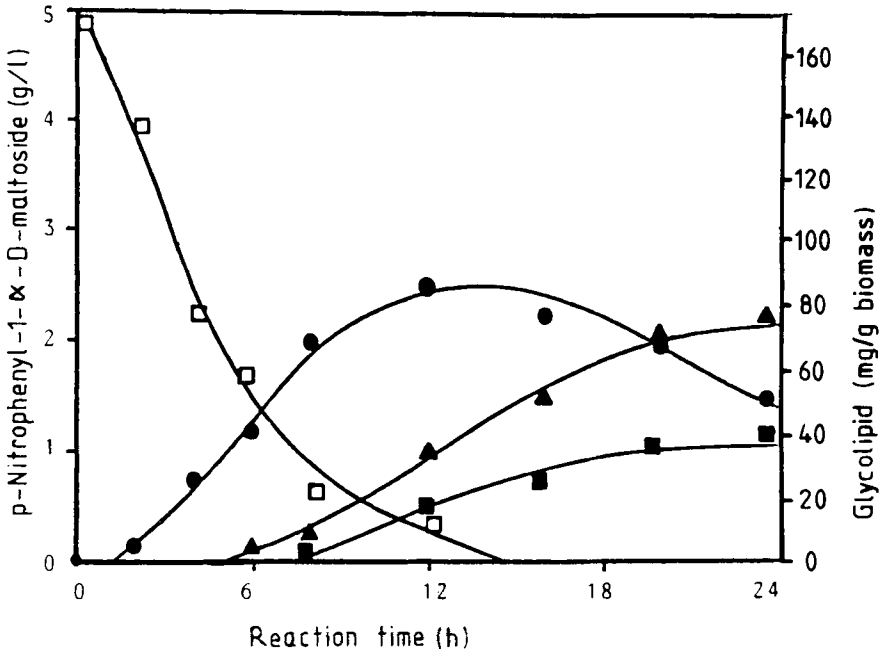

FIG. 5. Esterification of p-nitrophenyl-1-α-D-maltoside by resting whole cells of *Arthrobacter sp.* (after growth on glucose). Conditions: 100 ml 0.08 M phosphate buffer, pH 5.8, 11.5 g/l biomass (dry weight), 5 g/l p-nitrophenyl-1-α-D-maltoside, 30°C. (-□-), p-nitrophenyl-1-α-D-maltoside; (-●-), p-nitrophenyl-1-α-D-maltose-monocorynomycolate; (-▲-), maltose-monocorynomycolate; (-■-), p-nitrophenyl-l-α-D-glucose-monocorynomycolate.

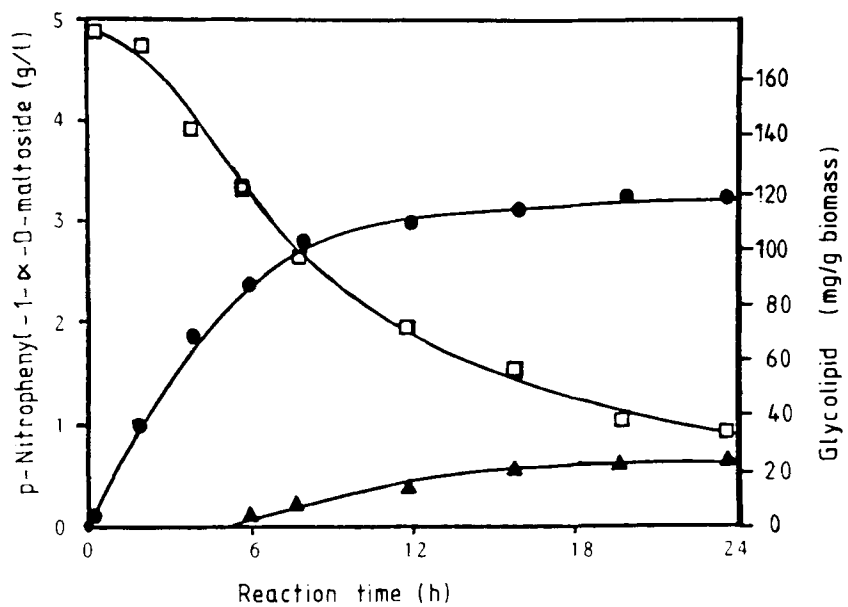

FIG. 6. Esterification of p-nitrophenyl-1-α-D-maltoside by cell debris of *Arthrobacter sp.* (after growth on glucose). Conditions: 100 ml 0.08 M phosphate buffer, pH 5.8, originally 11 g/l biomass (dry weight) before cell disruption, 5 g/l p-nitrophenyl-1-α-D-maltoside, 30°C. (-□-), p-nitrophenyl-1-α-D-maltoside; (-●-), p-nitrophenyl-1-α-D-maltose-monocorynomycolate; (-▲-), maltose-monocorynomycolate.

in the following studies cell particles bearing both esterase and glucose-corynomycolate were used in comparison to whole cells.

In these experiments the p-nitrophenyl-derivative of maltose served as acceptor molecule for the corynomycolic acid. Figure 5 and 6 show the concentration profiles of substrate and products. In the case of whole cells (Fig. 5), the substrate concentration decreased almost linearly from five g/l to about 0.6 g/l within the first eight hr and was reduced to zero after 16 hr. The formation of p-nitrophenyl-maltose-monoester amounted to only about 90 mg/g biomass or one g/l. After 12 hr the monoester concentration decreased.

The kinetics of the esterification by cell debris (Fig. 6) were as follows: The substrate consumption rate was only 2.3 g/l within the first eight hr. After 24 hr the substrate concentration was reduced to one g/l. The initial production of the p-nitrophenyl-1-α-D-maltose-monocorynomycolate amounted to more than one g/l or about 120 mg/g biomass (that means before cell disruption); thus, the primary product/substrate ratio was improved by cell disruption.

Concerning the reaction with cell particles, no p-nitrophenyl-glucose-ester was synthesized from p-nitrophenyl-1-α-D-maltoside in contrast to the whole cell experiments. This fact indicated that the cell particles were lacking the enzyme responsible for the cleavage of the α-1,4-linkage between the two glucose units of the substrate.

Concerning the cleavage of the p-nitrophenyl group from the substrate, no measurements were performed. The yellow color of the reaction batches and the production of the maltose-monocorynomycolate, however, were evidence enough for the presence of 1-α-glucosidase activity. The amount of maltose-ester was higher using whole cells than cell debris, indicating that a portion of enzyme activity was lost by cell disruption.

Influence of C1-substituents on sugar-corynomycolate production. Applying 2D-^{1}H-NMR measurements to elucidate new disaccharide lipids, we ascertained that the esterification of disaccharides with two free C6-positions always began at the C6 carbon atom of the nonreducing end of the molecule. Therefore, disaccharides were tested after the reducing sugar moiety had been converted into a nonreducing one. Palatinitol, a mixture of 1-0-(α-D-glucopyranosyl)-D-sorbitol (GPS) and 1-0-(α-D-glucopyranosyl)-D-mannitol (GPM), and maltitol were esterified by resting cells of *Arthrobacter sp.* DSM 2567. The kinetics of ester formation is compared with that of maltose and cellobiose in Figure 7.

In the case of esterification of GPM and maltitol, the total absence of monocorynomycolates was significant; the formation of dicroynomycolates started after four hr incubation, leading to 180 mg/g biomass at 24 hr. Esterification of GPS couldn't be observed; this fact indicates that the esterase exhibits a high enantio-selectivity.

The conversion of maltose showed the expected kinetics. The maltose-monocorynomycolate production was initiated after four hr and reached its maximum after 18 hr, two hr before the diester production began.

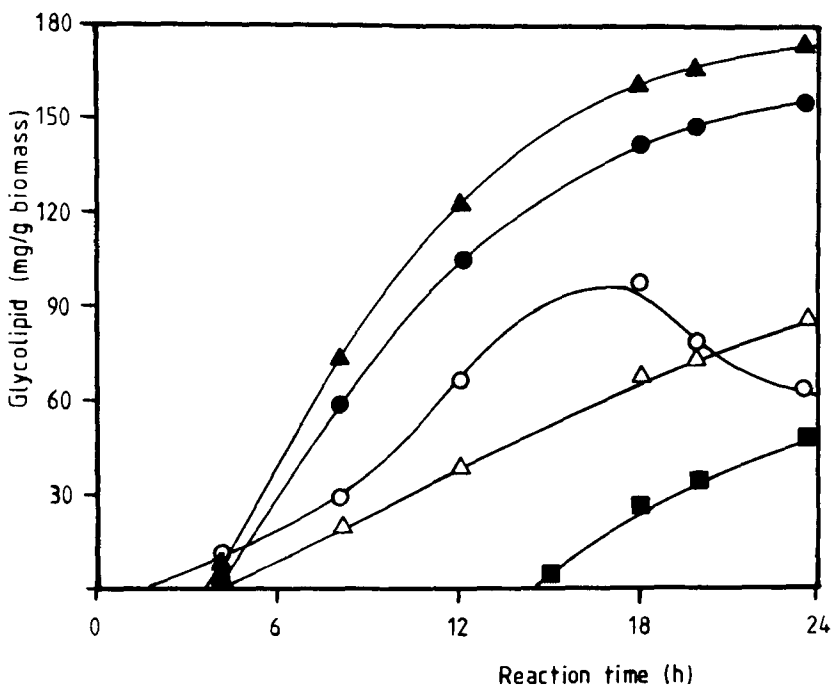

FIG. 7. Conversion of maltitol, 1-0-(α-D-glucopyranosyl)-D-mannitol (=GPM), maltose and cellobiose, resp., with resting cells of *Arthrobacter sp.* (after growth on glucose). Conditions: 100 ml 0.08 M phosphate buffer, pH 6.3, 12 g/l biomass, 30 g/l of the above substrates, 30°C. (-▲-), GPM-dicorynomycolate; (-●-), maltitol-dicorynomycolate; (-○-), maltose-monocorynomycolate; (-■-), maltose-monocorynomycolate; (-■-), maltose-dicorynomycolate; (-△-), cellobiose-monocorynomycolate.

Using cellobiose as disaccharide only the C-6-monoester was produced.

Summarizing these results on the enzymatic disaccharide esterification we can conclude:

(i) In the presence of two nonreducing molecular ends with two primary alcoholic groups at C-6 positions, both positions were substituted simultaneously leading directly to the dicorynomycolates.

(ii) If only one nonreducing moiety was present the C-6 position of this sugar was esterified first; the diester production followed later.

(iii) The esterification of the reducing molecular end leading to the diester was not possible or restricted to a lower level, if the reducing moiety of the disaccharide was bound by a β1,4 linkage. This was in agreement with our fruitless studies on the esterification of galactose, lactose, lactulose, melibiose, stachyose and raffinose (Table 2), which have a β-4-OH-group at the nonreducing end.

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