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BIOCATALYSIS

Effect of growth media on cell envelope composition and nitrile hydratase stability in *Rhodococcus rhodochrous* strain DAP 96253

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Abstract *Rhodococcus* is an important industrial microorganism that possesses diverse metabolic capabilities; it also has a cell envelope, composed of an outer layer of mycolic acids and glycolipids. Selected *Rhodococcus* species when induced are capable of transforming nitriles to the corresponding amide by the enzyme nitrile hydratase (NHase), and subsequently to the corresponding acid via an amidase. This nitrile biochemistry has generated interest in using the rhodococci as biocatalysts. It was hypothesized that altering sugars in the growth medium might impact cell envelope components and have effects on NHase. When the primary carbon source in growth media was changed from glucose to fructose, maltose, or maltodextrin, the NHase activity increased. Cells grown in the presence of maltose and maltodextrin showed the highest activities against propionitrile, 197 and 202 units/mg cdw, respectively. Stability of NHase was also affected as cells grown in the presence of maltose and maltodextrin retained more NHase activity at 55 °C (45 and 23 %, respectively) than cells grown in the presence of glucose or fructose (19 and 10 %, respectively). Supplementation of trehalose in the growth media resulted in increased NHase stability at 55 °C, as cells grown in the presence of glucose retained 40 % NHase activity as opposed to 19 % without the presence of trehalose. Changes in cell envelope components, such mycolic acids and glycolipids, were evaluated by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC), respectively. Changing sugars and the addition of inducing components for NHase, such as cobalt and urea in growth media, resulted in changes in mycolic acid profiles. Mycolic acid content increased 5 times when cobalt and urea were added to media with glucose. Glycolipids levels were also affected by the changes in sugars and addition of inducing components. This research demonstrates that carbohydrate selection impacts NHase activity and stability. Cell envelope components such as mycolic acids are also influenced by sugars and inducers such as cobalt and urea. This is information that can be useful when implementing rhodococcal catalysts in industrial applications.

Keywords *Rhodococcus* · Nitrile hydratase · Cell envelope · Mycolic acids

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Introduction

Members of the genus *Rhodococcus* are nocardioform actinomycetes, and are members of the well-characterized suprageneric taxon mycolata which includes *Mycobacterium, Nocardia, Corynebacterium*, and *Rhodococcus* all of which possess a unique cell envelope [8]. The outer envelope layer is composed of mycolic acids [10], which are α-alkyl, β-hydroxy fatty acids, attached to an arabinogalactan structure which in turn is linked to peptidoglycan [3]. Mycolic acids can either be bound or 'free'. In Sutcliffe's model [19, 20] the outer layer of the cell envelope has free mycolic acids that are intercalated with bound mycolic acids acting as plugs forming an outer lipid bilayer. The free mycolic acids are linked to a trehalose moiety (as trehalose monomycolates or trehalose dimycolates).

Trehalose is a non-reducing disaccharide of D-glucose that is found in many plants, insects, and microorganisms and has been shown to preserve the activity of proteins under stressful conditions such as dehydration or heat [17, 22].

There is significant evidence that growth on hydrocarbons affects the envelope mycolic content of *Rhodococcus*. Wick et al. [21] showed that envelope mycolic acid profiles were altered when the growth substrate was changed from glucose to alkane. Sokolovska et al. [16] reported that growth on hydrophobic substrates (e.g., alkanes and polycyclic aromatic hydrocarbons) led to a shift to more hydrophobic mycolic acids; researchers proposed a hypothetical mechanism for changes in hydrophobicity which may involve changing the ratio of bound to free mycolic acids in the cell wall. Stratton et al. [18] observed significant differences in the carbon length and the level of saturation of mycolic acids between cells of *Rhodococcus* sp. strain 11R grown on glucose or Tween® 80.

Rhodococcus is an important industrial organism that is used in the biotransformation of nitriles. Nitriles can be transformed to the corresponding amide by nitrile hydratase (NHase) [9, 23], and the amides are converted to their corresponding acid and ammonia by amidase. Nagasawa et al. [11, 12] showed that NHase in *Rhodococcus* J1 was induced by the presence of cobalt ions and urea. NHase from *Rhodococcus* is currently employed in the production of acrylamide and nicotinamide [23]. Recently, we showed that *Rhodococcus rhodochrous* DAP 96253 grown in the presence of inducers was able to delay the ripening of fruit [13, 14].

In order to apply *Rhodococcus* to industrial processes, desired enzyme levels need to be relatively high and stable. In this work, we show that supplementation of different sugars such as glucose, fructose, maltose, or maltodextrin in growth media influences both NHase activity and stability in *R. rhodochrous* strain DAP 96253. We also examined the effects of changes in growth media supplementation on cell envelope components, such as mycolic acids and glycolipids.

Materials and methods

Growth media and culture conditions

Rhodococcus rhodochrous strain DAP 96253 stored as glycerol stocks (at −80 °C) were revived by adding the entire contents of a 1-ml vial to 50 ml Nutrient Broth in a 250-ml flask, and incubated at 30 °C for 3 days while shaking at 180 rpm. After 3 days, cells were transferred to Nutrient Agar (NA) plates and checked for purity. Cells from NA plates were used to inoculate Yeast Extract Malt Extract Agar [YEMEA (4 g yeast extract, 10 g malt extract, 4 g glucose and 20 g agar in 1 l) [4].

The base YEMEA medium was modified by replacing the glucose with fructose, maltose, or maltodextrin (4 g/l). In addition the modified YEMEA also contained cobalt chloride ($CoCl_2 \cdot 6H_2O$, 0.201 g/l, 10 ppm cobalt) and urea (7.5 g/l) as inducers.

NHase assay

A unit of NHase activity was defined as the amount of NHase that catalyzed the conversion of 1 μM of nitrile to 1 μM amide in 1 min, at pH 7.2 and 30 °C. NHase activity was determined using 1,000 ppm acrylonitrile (AN) (Aldrich Chemical Company, Milwaukee, WI) as the source of nitrile.

NHase activity was determined using the method outlined by Pierce et al. [13]. The cells (40–50 mg, packed wet wt) were first suspended in 1 ml of 50 mM phosphate buffer (pH 7.2) in a 1.5-ml Eppendorf® tube and combined with 9 ml of buffer and nitrile added to achieve 1,000 ppm. This suspension was mixed and stopped rapidly by the addition of 200 μl of 2 N sulfuric acid, after mixing for 1 min; 50 μl of 8 N sodium hydroxide was added to neutralize the acid. Duplicate 1-ml samples were placed in separate 1.5-ml Eppendorf® tubes and centrifuged for 2 min at 13,000 rpm to remove cells, and the supernatant was transferred to fresh Eppendorf ® tubes.

To ensure complete amide conversion to acid and ammonia, commercial amidase (1,000 units/440 μl, stored at −20 °C, Sigma-Aldrich, St. Louis, MO) was added to the mixture. A working amidase solution was prepared by making a 1:50 dilution of the original amidase solution; 10 μl of this working solution was added to each sample for the amide conversion to acid and ammonia, followed by vortexing for 30 s. Samples were incubated at 37 °C for 30 min.

Ammonia was determined by modification of the method reported by Fawcett and Scott [6]. The converted amide solution was transferred to 15-ml glass test tubes to which 2 ml of sodium phenate was added followed by the addition of 3 ml 0.1 % aqueous sodium nitroprusside (Sigma-Aldrich, St. Louis, MO) and 3 ml 0.02 N sodium hypochlorite (Clorox, 6.15 %); the resulting solution was mixed by vortexing lightly. Color development was achieved by incubation of the tubes in the dark at 27 °C for 30 min. After color development the tubes were vortexed again and the OD read at 630 nm for 10 s using a microplate reader (Wallac Victor® 1420 multilabel counter, Perkin Elmer Life Sciences, Shelton, CT). Each sample was read in triplicate and the data averaged.

Stability tests

Cells (50 mg, wet wt) were placed in 1 ml 50 mM phosphate buffer and allowed to equilibrate at 10 °C, 55 °C for 1 h. An initial NHase activity was determined as described above by reacting the cells with 1,000 ppm AN at room

temperature. The stability of NHase was assessed by measuring activity at different time points.

Mycolic acid extraction

The Center for Disease Control (CDC) standardized method for the extraction and subsequent HPLC analysis of mycolic acids for the identification of *Mycobacteria* was employed as follows [2]. Chemicals used were purchased from Sigma-Aldrich (St. Louis, MO). Two loopfuls of *R. rhodochrous* strain DAP 96253 were scraped from YEMEA plates, combined, and then mixed with 2 ml of the saponification reagent in a 15-ml tube. The saponification reagent was prepared by adding 200 g of potassium hydroxide to 400 ml deionized water followed by the addition of 400 ml of methanol. The tubes were covered, mixed vigorously for 30 s, the tops wrapped in foil, and then autoclaved for 80 min at 121 °C. Upon cooling, 1.5 ml of the acidification reagent (50 % solution of hydrochloric acid) was added to each tube and then mixed for 30 s by shaking at room temperature. The acidified mixture was extracted twice with 1 ml of dichloromethane (DCM). The pooled DCM extracts were placed in a heating block (VWR Scientific, Batavia, IL) at 85 °C for 15 min. Then, 100 μl of the potassium bicarbonate reagent [prepared by adding 4 g of potassium bicarbonate to 196 ml of deionized water/methanol (1:1)] was added to the extracts and they were redried under nitrogen as above. After this redrying cycle, 1 ml of DCM and 50 μl of *p*-bromophenacyl-8 reagent [*p*-bromphenacyl bromide (0.1 mmol/ml) and dicyclohexyl-18-crown-6 ether (0.005 mmol/ml) in acetonitrile (Pierce Chemical Company, Rockford, IL)] were added to the samples. The samples were vortexed for 30 s then placed in the heating block at 85 °C for 25 min after which the tubes were removed, allowed to cool, and then 1 ml of clarification reagent was added followed by vortexing for 20 s. Clarification reagent was prepared by adding 100 ml of the acidification reagent to 100 ml of methanol. The organic layer was removed with a glass Pasteur pipette and transferred to a clean test tube then evaporated to dryness under nitrogen using a heating block at 85 °C. The samples were capped tightly and stored at 4 °C in the dark [1, 5].

HPLC analysis

Changes in mycolic acid content were documented by examining the HPLC profiles of the extracted mycolic acids. In the CDC method [2] used for the identification of *Mycobacteria* through HPLC profiles, both a negative control for mycolic acid production (*Candida albicans* CA30) and a positive control (*Mycobacterium intracellulare* ATCC 13950) were used in analysis and in addition, a high molecular weight (HMW) internal standard was also incorporated into each sample. Samples were analyzed in duplicate.

Dried samples of derivatized mycolic acids were re-solubilized in 500 μl of a solution containing 5 mg of HMW standard [C110 (Corixa Corporation, Hamilton, MT)] in 100 ml DCM. HPLC analysis was performed using a Series 200 HPLC instrument (Perkin Elmer, Shelton, CT) equipped with a UV detector. A Novapak® RP C18 column (3.9 mm × 300 mm, 4 μm, 60 A, Waters, Milford, MA) was used. The mobile phase comprised methanol and DCM in a step gradient: 0–13 min, 0–10 %; 13–17 min, 10–25 %; 17–34 min, 25–75 %; 34–41 min, 30–70 %; 41–45 min, 100–0 %. The flow rate was 1 ml/min and the detector was set at 254 nm [15].

Extraction of total lipids

Cells for lipid extraction were scraped from YEMEA plates then weighed. Approximately 50 mg of cells (packed wet wt) was suspended in 5 ml of chloroform/methanol (2:1) in a 20-ml screw cap glass vial and incubated for 1 h at room temperature at 120 rpm. The samples were centrifuged at 3,000 rpm for 15 min. The above process was repeated two more times but with using chloroform/methanol (1:2) in the first repeat and then chloroform/methanol (1:1) in the second repeat [15]. All three extracts were pooled and dried under nitrogen at 65 °C and then resuspended in 100 μl of chloroform. Multiple small aliquots (~20 μl) were spotted onto a TLC plate (Merck, silica gel 60, 10–20 cm, 0.25 mm thickness) until a total of 100 μl of each sample was spotted. Trehalose dimycolate standard (2.5 mg, Sigma-Aldrich, St. Louis, MO) was also spotted onto the plates. The TLC plates were developed in chloroform/methanol/acetone/acetic acid (90:10:6:1) and then sprayed with a 15 % ethanolic solution of 1-naphthol (Aldrich, Milwaukee, WI). The glycolipids spots (purple) were visualized following heating at 100 °C for 3–6 min.

Cells for lipid extraction were scraped from YEMEA plates then weighed. Approximately 200 mg of cells (packed wet wt) was suspended in 10 ml of chloroform/methanol (2:1). The same procedure was followed as in part A. The TLC plates were developed in chloroform/methanol/acetic acid (60:40:10), followed by spraying with a 15 % ethanolic solution of 1-naphthol (Aldrich, Milwaukee, WI). The glycolipids spots (purple) were visualized following heating at 100 °C for 3–6 min.

Results

Effect media composition on NHase activity

The NHase activity against selected nitriles was determined (Table 1). Substrates used were acrylonitrile (AN),

Table 1 NHase activity (units/mg cdw) against selected nitriles for *R. rhodochrous* DAP 96253 grown on YEMEA with inducers and supplementation of different sugars

Substrate	Glucose	Fructose	Maltose	Maltodextrin
AN	100	120	133	150
PN	125	128	197	202
BN	18	21	35	41
CrN	13	9	12	9

YEMEA was supplemented with different sugars, such as glucose, fructose, maltose, and maltodextrin, and inducers cobalt and urea. One unit was defined as the amount of NHase that catalyzed the conversion of 1 μM of nitrile to 1 μM of amide in 1 min, at pH 7.2 and 30 °C

AN acrylonitrile, *PN* propionitrile, *BN* butyronitrile, *CrN* crotononitrile

propionitrile (PN), butyronitrile (BN), and crotonitrile (CrN). NHase activities to BN and CrN were significantly lower than the NHase activity to AN and PN. Induced cells grown on maltose and maltodextrin had the highest NHase activity to PN out of all the substrates examined. Table 1 shows that supplementing different sugars in the growth media impacts NHase activity (Table 2 shows the statistical evaluation of the data).

Table 2 Statistical evaluation of NHase activities (units/mg cdw) of *R. rhodochrous* DAP 96253 grown on YEMEA supplemented with different sugars and inducers against acrylonitrile

Supplement	*p* value	SD
F, Co, U[a]	0.001	11.01
M, Co, U[a]	0.018	23.87
MD, Co, U[a]	0.012	33.55
M Co U[b]	0.024	13.60
MD Co, U[b]	0.010	23.11
MD, Co, U[c]	0.002	12.91

G glucose, *F* fructose, *M* maltose, *MD* maltodextrin, *Co* cobalt, *U* urea, *SD* standard deviation

NHase activities compared with NHase activity of *R. rhodochrous* DAP 96253 grown on YEMEA supplemented with [a] glucose, cobalt, and urea; [b] fructose, cobalt, and urea; [c] maltose, cobalt, and urea

NHase stability

The stability of NHase in *R. rhodochrous* DAP 96253 cells was assessed at 10 °C and at 55 °C after growth on YEMEA supplemented with different sugars and inducers. Table 3 shows NHase stability over a 30-h period at 10 °C. Cells grown on YEMEA supplemented with fructose cobalt and urea lost the greatest percentage of activity (44 %), whereas cells grown on YEMEA supplemented with maltose or maltodextrin retained the most activity with only a 16 % loss.

NHase stability at 55 °C was also influenced by changes in media composition, with cells grown in the presence of maltose and maltodextrin retaining the most NHase activity (Table 4). The addition of trehalose to YEMEA supplemented with different sugars resulted in an increased stability of NHase, e.g., cells grown on YEMEA supplemented with fructose, cobalt, and urea initially lost 90 % of NHase activity within 45 h at 55 °C, but cells grown in the presence of trehalose lost only 68 % of NHase activity.

Table 3 NHase stability in *R. rhodochrous* DAP 96253 cells maintained at 10 °C for 30 h after growth on YEMEA supplemented with different sugars and inducers

Supplement	NHase activity lost at 10 °C (%)
G, Co, U	26
F, Co, U	44
M, Co, U	16
MD, Co, U	16

Data were compared with those at $t = 0$ and 30 °C

G glucose, *F* fructose, *M* maltose, *MD* maltodextrin, *Co* cobalt, *U* urea

Mycolic acid profiles

Examination of patterns of extracted mycolic acid and relative abundance showed that supplementation of different sugars and inducers (cobalt and urea) in YEMEA markedly affected the cell envelope mycolic acid composition. Mycolic acid profiles for non-induced cells of *R. rhodochrous* strain 96253 grown on YEMEA supplemented with different sugars are shown in Fig. 1. Mycolic acid profiles for cells grown on the same media supplemented with the different sugars, but now also containing cobalt and urea to induce NHase and amidase, are shown in Fig. 2.

From Figs. 1 and 2, it is apparent that there were significant differences in mycolic acid profiles/content for cells grown in the presence of glucose, maltose, and maltodextrin when inducers (cobalt and urea) were present in the growth media. Significant changes in mycolic acid profiles/content were not noted for the fructose-supplemented media whether induced or not.

Table 4 NHase stability at 55 °C after growth on YEMEA supplemented with different sugars and inducers

Supplements in YEMEA	NHase activity lost (%)	Statistical evaluation *p* value	SD
G, Co, U	81		
F, Co, U	90	0.035	4.14
M, Co, U	55	0.030	18.71
MD, Co, U	77	0.085	13.73
G, Co, U, Tre	60	0.037	9.48
F, Co, U, Tre	68	0.002	6.89
M, Co, U, Tre	41	0.004	22.19
MD, Co, U, Tre	54	0.003	17.81

All data were compared with the NHase activity of cells grown on YEMEA with glucose, cobalt, and urea after 45-h incubation at 55 °C

G glucose, *F* fructose, *M* maltose, *MD* maltodextrin, *Co* cobalt, *U* urea, *SD* standard deviation

Extractable mycolic acid content also assessed by the summation of all peak areas between retention time (RT) 23 and 30 min followed by normalization of the values against glucose without cobalt and urea (Table 5). The addition of cobalt and urea to YEMEA supplemented with glucose or fructose significantly increased the mycolic acids content.

Analysis of glycolipids

It was hypothesized that in addition to changes in mycolic acid content changes in the glycolipid content of the cell envelope might also occur. Total lipids were extracted from *Rhodococcus rhodochrous* strain DAP 96253, separated on silica gel plates [15], and visualized by spraying with 1-naphthol followed by heating as shown in Fig. 3. Not surprisingly, none of the visualized lipid spots from the extracts corresponded to the position for the standard glycolipid used [trehalose dimycolate (TDM) purified from *Mycobacterium tuberculosis*]; it could be inferred that the spots observed were glycolipids as the standard also turned purple upon spraying with 1- naphthol. Cells that were uninduced and grown on YEMEA supplemented with maltose or maltodextrin did not show any glycolipid spots. However, cells grown on YEMEA supplemented with glucose or fructose showed a single glycolipid spot. The supplementation of cobalt and urea resulted in a significant increase in glycolipids for cells grown in the presence of glucose and fructose.

Figure 4 shows that the incorporation of cobalt and/or urea in growth media resulted in changes in cell envelope glycolipids. Two additional glycolipids were observed when cobalt was added and one additional glycolipid when urea was added. The supplementation of both cobalt and urea in the same media resulted in the loss of the third glycolipid spot observed with cobalt only supplementation.

Discussion

Propionitrile (PN) was the best substrate for the NHase from *Rhodococcus* sp. DAP 96253, followed by acrylonitrile (AN), butyronitrile (BN), and crotononitrile (CrN). Growth of *R. rhodochrous* DAP 96253 on YEMEA supplemented with different carbohydrates resulted in statistically significant differences in NHase activity against AN and PN. This showed that supplementation of different sugars or carbohydrates such as maltodextrin influences NHase activity.

Supplementation of growth media with different primary sugars not only affected NHase activity but also affected the stability of the enzyme. NHase in cells of *R. rhodochrous* DAP 96253 grown on maltose and maltodextrin was statistically more stable at 55 °C than NHase in cells grown in the presence of glucose or fructose.

Supplementation with trehalose significantly increased the thermostability of NHase in cells grown on YEMEA supplemented with glucose or fructose, cobalt, and urea (Table 4).

Previously, researchers had shown that shifting to a hydrocarbon-based growth media influenced mycolic acid composition in selected members of *Rhodococcus*. Changes in HPLC mycolic acid profiles were observed when hydrocarbons replaced glucose as the carbon source, or when hydrocarbons of various chain lengths were used [16]. Our study clearly shows that supplementing fructose, maltose, or maltodextrin for glucose resulted in significant changes in cell envelope mycolic acids. The addition of metals such as cobalt (NHase inducer) and urea (amidase inducer) to the growth medium also resulted in significant changes in mycolic acid profiles suggesting that other components of growth media, such as metals, can have an impact on the mycolic acids content of *R. rhodochrous* DAP 96253.

The examination of mycolic acid profiles is a powerful and relatively easy tool that has been used by the CDC in

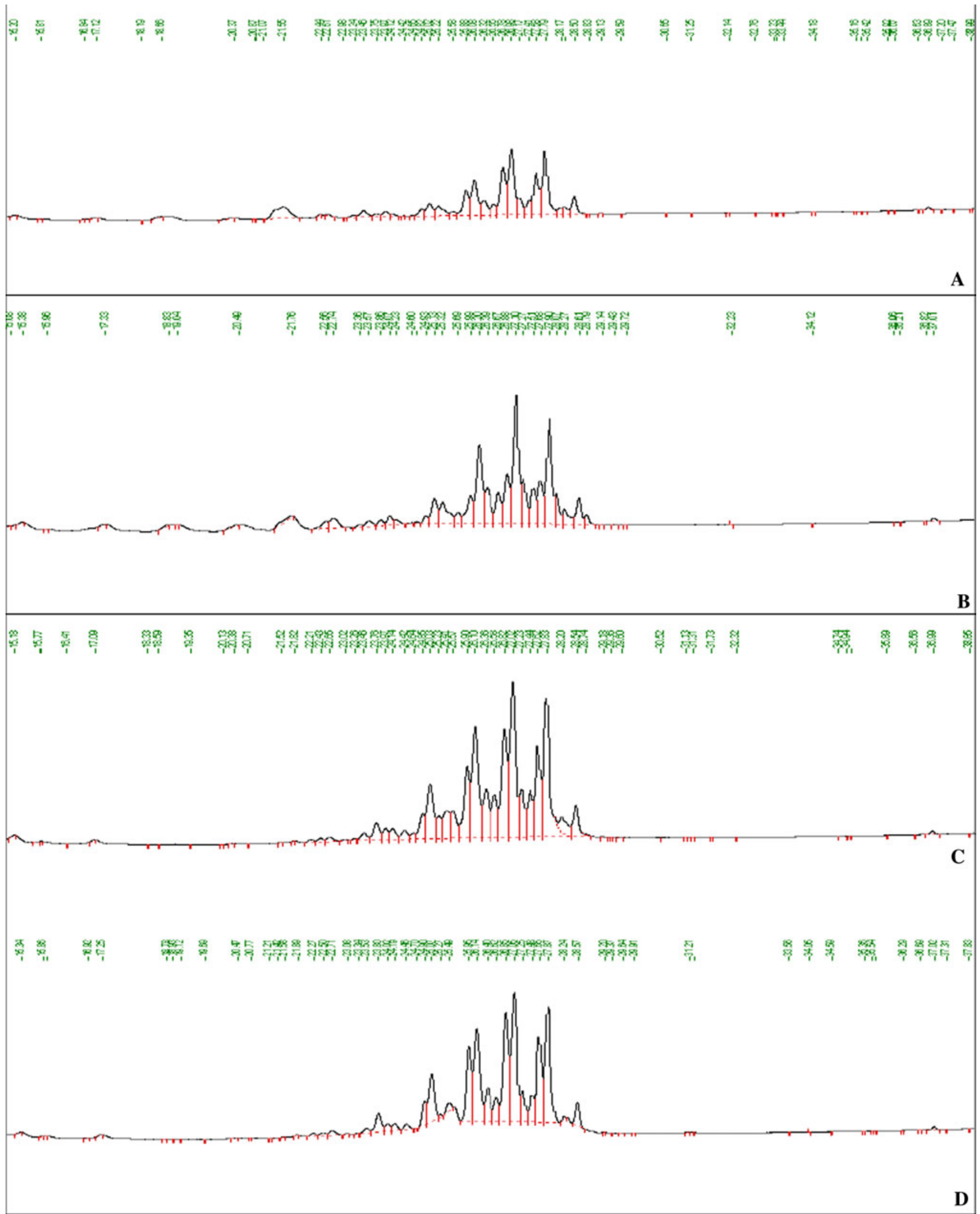

Fig. 1 Chromatograms of mycolic acid extracted from *Rhodococcus rhodochrous* strain DAP 96253 grown on YEMEA supplemented with **a** glucose, **b** fructose, **c** maltose, **d** maltodextrin. *X*-axis shows time 20–35 min, *Y*-axis response (mV)

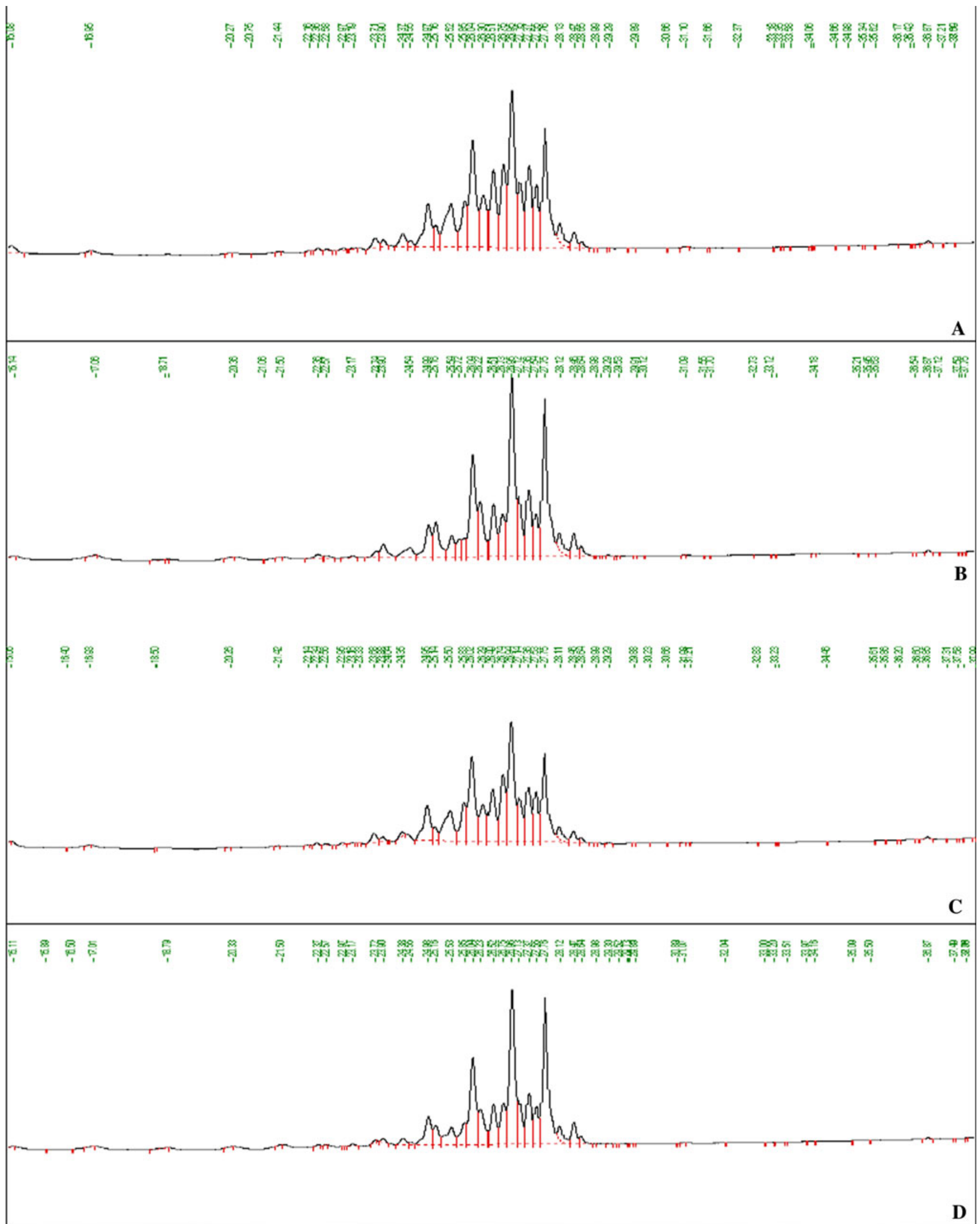

Fig. 2 Chromatograms of mycolic acid extracted from *R. rhodochrous* strain DAP containing cobalt and urea supplemented with **a** glucose, **b** fructose, **c** maltose, **d** maltodextrin. *X*-axis shows time 20–35 min, *Y*-axis response (mV)

Table 5 Normalized values for mycolic acid content between retention times of 23 and 30 min

Media	Mycolic acid content
G	100
F	455
M	125
MD	490
G, Co, U	500
F, Co, U	405
M, Co, U	485
MD, Co, U	330

G glucose, *F* fructose, *M* maltose, *MD* maltodextrin, *Co* cobalt, *U* urea

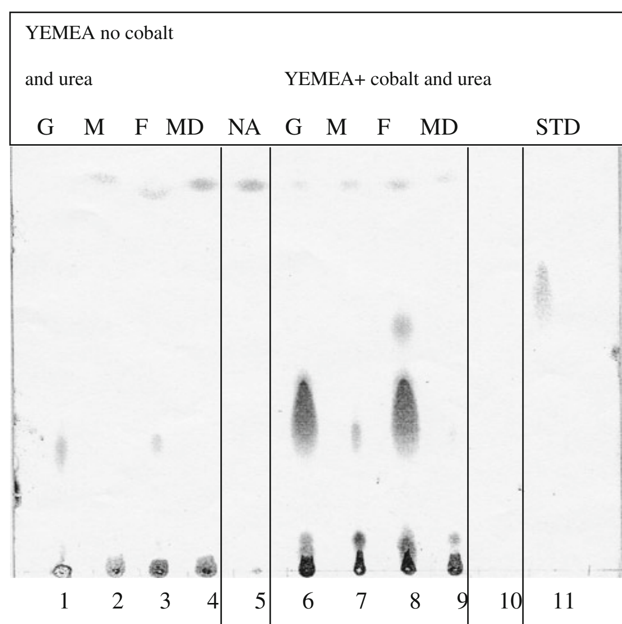

Fig. 3 TLC of total lipid extracts (extracted and analyzed using method A) sprayed with 1-naphthol to visualize glycolipids. *Rhodococcus rhodochrous* strain DAP 96253 was grown on YEMEA supplemented with glucose (*lane 1*), YEMEA supplemented with maltose (*lane 2*), YEMEA supplemented with fructose (*lane 3*), YEMEA supplemented with maltodextrin (25 DE) (*lane 4*), nutrient agar (*lane 5*), YEMEA supplemented with glucose, cobalt, and urea (*lane 6*), YEMEA supplemented with maltose, cobalt, and urea (*lane 7*), YEMEA supplemented with fructose, cobalt, and urea (*lane 8*), YEMEA supplemented with maltodextrin, cobalt, and urea (*lane 9*); *lane 10* was blank (no sample) and *lane 11* contained trehalose dimycolate (TDM) purified from *Mycobacterium tuberculosis*

species identification of *Mycobacterium*, in differentiating *Rhodococcus* species, and in distinguishing members of the mycolata taxon [1, 2]. Media selection would be extremely important when mycolic acid profiles are being used in the above applications as changes in sugars in the media can affect mycolic acid profiles. The supplementation of different sugars and inducers (cobalt and urea) in growth media also affected the type and quantity of glycolipids in the cell envelope. On the basis of the changes observed, more detailed experimentation is warranted in order to identify the mycolic acids and lipids present in the cell envelope of NHase induced *Rhodococcus*.

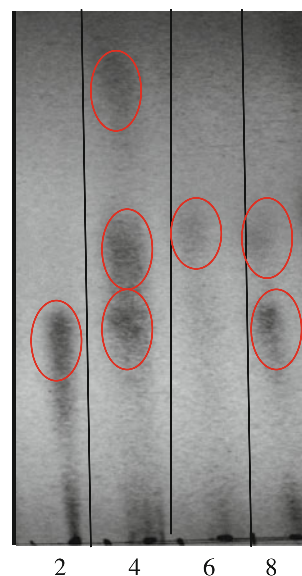

Fig. 4 TLC of total lipid extracts (extracted and analyzed using method B) sprayed with 1-naphthol to visualize glycolipids. *Rhodococcus rhodochrous* strain DAP 96253 was grown on YEMEA supplemented with glucose only (*lane 2*), YEMEA supplemented with glucose and cobalt (*lane 4*), YEMEA supplemented with glucose and urea (*lane 6*), YEMEA supplemented with glucose, cobalt, and urea (*lane 8*)

Gebhardt et al. [7] showed that growth on minimal media supplemented with sucrose did not result in mycolic acid production, but with the addition of trehalose, mycolic acids were produced and found linked to trehalose in extractable lipids. Our experimental evidence strongly links trehalose availability to mycolic acid production. The supplementation of glucose, fructose, maltose, or maltodextrin in YEMEA resulted in significant changes in the levels of cellular trehalose (data not shown). These changes in cellular trehalose may have contributed to the changes seen in mycolic acid and glycolipid content in the cell envelope of *R. rhodochrous* strain DAP 96253.

This research showed that altering growth media, specifically sugars, affected both the NHase initial activity and stability of NHase in *R. rhodochrous* DAP 96253. This work also shows that achieving increased enzyme activity and stability is not solely dependent on actual enzyme production, but also may influence other aspects of the cell, such as the cell envelope.

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