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The Effect of Media Composition on Nitrile Hydratase Activity and Stability, and on Cell Envelope Components of Rhodococcus DAP 96253

Trudy-Ann Marie Tucker

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**EFFECT OF CHANGES IN MEDIA COMPOSITION ON NITRILE
HYDRATASE ACTIVITY AND STABILITY AND ON CELL ENVELOPE
COMPONENTS OF *RHODOCOCCUS* SP DAP 96253**

by

TRUDY-ANN TUCKER

Under the Direction of George E. Pierce

ABSTRACT

Rhodococcus is an important industrial organism that possesses diverse metabolic capabilities, it also has a unique cell envelope, composed of an outer layer of mycolic acids and glycolipids (free or bound lipids generally linked to the sugar trehalose). *Rhodococcus* is able to transform nitriles to the corresponding amide by the enzyme Nitrile Hydratase (NHase), therefore rhodococcal cells can be utilized as biocatalysts in the detoxification of nitrile waste water or in the production of industrially important amides such as acrylamide. However, the NHase within the native cells must be stable with high activity.

This research examined how NHase activity and stability can be increased in native cells by changing growth media composition, the impact on the rhodococcal cell envelope was also studied.

Growth media composition was altered by supplementing different sugars such as fructose, maltose or maltodextrin to replace glucose in rich solid media containing cobalt and urea for induction of NHase. The supplementation of maltose or maltodextrin resulted in significantly higher NHase activities and greater NHase stability at 55°C. The supplementation

of these different sugars was shown to alter cellular and lipid bound trehalose levels, a sugar known to stabilize proteins and a component of the rhodococcal cell envelope. Cells that had higher levels of cellular trehalose had significantly greater NHase stability at 55°C.

The effect of the different sugar supplements and inducers of NHase, such as cobalt, on cell envelope components such as mycolic acids and glycolipids were examined by High Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC). The results showed that changes in mycolic acids and glycolipids occurred when the cells were grown in the presence of different sugar supplements and when the cells were induced for NHase.

Susceptibility of *Rhodococcus* sp DAP 96253 to different antibiotics was examined to indicate if changes were occurring in the cell envelope. Differences in antibiotic susceptibility were observed when the cells were grown on media with different sugar supplements and when the cells were induced for NHase. In the presence of cobalt *Rhodococcus* sp DAP 96253 showed a significant increase in sensitivity to antibiotics.

Changes in growth media composition influences the cell envelope of *Rhodococcus* sp DAP 96253 and also affects NHase activity and stability. Therefore, achieving increased enzyme activity and stability is not entirely dependent on the actual enzyme, but is related to other aspects of the cell, such as the cell envelope and metabolites of the cell.

INDEX WORDS: *Rhodococcus*, Nitrile Hydratase, Cell envelope, Mycolic acids, Growth media, Trehalose

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by

TRUDY-ANN TUCKER

A Dissertation Submitted in Partial Fulfillment of the Requirement for the degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2007

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Trudy-Ann Tucker
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LIST OF ABBREVIATIONS

| | |
|---------------|--------------------------------------------|
| AN..... | Acrylonitrile |
| AMD..... | Acrylamide |
| AA..... | Acrylic acid |
| ACN..... | Acetonitrile |
| SN..... | Succinonitrile |
| FN..... | Fumaronitrile |
| NHase..... | Nitrile Hydratase |
| H-NHase..... | High molecular weight nitrile hydratase |
| L-NHase..... | Low molecular weight nitrile hydratase |
| ASN..... | Asparagine |
| AG..... | Arabinogalactan |
| LAM..... | Lipoarabinomannans |
| LM..... | Lipomannans |
| LG..... | Lipoglycans |
| CO..... | Cholesterol oxidase |
| S-layer..... | Surface layer |
| CD..... | Czapek Dox |
| GME..... | Glucose malt extract |
| YEMEA..... | Yeast extract malt extract agar |
| YEMEA NI..... | YEMEA non-induced |

| | |
|--------------|-------------------------------------|
| G..... | glucose |
| YEMEA-I..... | YEMEA induced |
| ME..... | Malt extract |
| YE..... | Yeast extract |
| DCM..... | Dichloromethane |
| TCA..... | Trichloroacetic acid |
| HMDS..... | Hexamethyldisiloxane |
| TMCS..... | Trimethylchlorosilane |
| FID..... | Flame ionizing detector |
| MIC..... | Minimum Inhibitory Concentration |
| ZOI..... | Zone of inhibition |
| PN..... | Propionitrile |
| BN..... | Butyronitrile |
| CrN..... | Crotononitrile |
| F..... | Fructose |
| M..... | Maltose |
| MD..... | Maltodextrin |
| Co..... | Cobalt |
| U..... | Urea |
| Tre..... | Trehalose |
| HMW..... | High molecular weight |

| | |
|----------|----------------------|
| LMW..... | Low molecular weight |
| TDM..... | Trehalose dimycolate |
| CDW..... | Cellular dry weight |

Introduction

Background

Nitrile compounds are numerous and widespread in the environment, produced by selected plants as cyanoglycosides, cyanolipids, ricinine, and phenylacetonitrile, (Conn, 1981) and as a result of certain industrial processes. Acrylonitrile (AN) is an important industrial compound. The world capacity for AN was reported in 1996 to be 4.3×10^6 tons per year. The United States manufactures 1.4×10^6 tons per year of AN (Weissermel and Arpe, 1997). AN is used to produce acrylamide (AMD), acrylic acid (AA), acrylic fibers, copolymer resins and nitrile rubbers (Pierce, 1999); it is manufactured by the Sohio/BP process which involves the direct ammoxidation of propylene by ammonia vapors in air using uranyl antimonite as a catalyst. The waste water generated from the Sohio process contains different nitriles, including acetonitrile (ACN), succinonitrile (SN), fumaronitrile (FN) and AMD. In addition, free cyanide is also present at varying concentrations (Pierce, US Patent, 1998). Pollak et al. (1991) showed that many nitriles are mutagens or carcinogens. The mechanism for nitrile toxicity is the inactivation of the respiratory system by binding of cytochrome-c-oxidase (Solomonson and Spehar, 1981). Nitriles such as acrylonitrile, acetonitrile, succinonitrile and fumaronitrile are serious health hazards and their treatment should be a primary concern.

AMD is also an important nitrile compound as it is a monomer for synthetic fibers and flocculant agents; about 200,000 tons are produced each year. AMD is synthesized conventionally by the hydration of acrylonitrile in the presence of copper catalysts. This process produces unwanted by-products and requires high temperatures, which increases

production costs. Furthermore, the catalyst is not easily regenerated (Nagasawa and Yamada, 1990).

Problems associated with the traditional manufacture of AN include high production costs and the generation of hazardous waste. The waste is not treated before disposal, this poses serious health hazard. The utilization of microorganisms to treat the waste (biodegradation) is an alternative (Aiken, 1993). This would remove or lower concentrations of hazardous nitriles present in the waste. Employing microorganisms in transformation reactions (biotransformation) is also an alternative to the traditional synthesis of AMD (Yamada and Kobayashi, 1996). *Rhodococcus* was shown to be a nitrile utilizing bacterium (Watanabe, 1987; Wyatt and Knowles, 1995) and can be used in biodegradation of nitrile waste waters and in the biotransformation of AN to AMD.

Metabolic diversity and uses of *Rhodococcus*

Members of the genus *Rhodococcus* are Gram-positive, non-motile, aerobic, chemoorganotrophic, pleomorphic rods which undergo oxidative metabolism and possess the capacity to form limited substrate mycelium (Goodfellow, 1989). Most rhodococci are capable of forming pigmented colonies that are red, orange or pink, in color due to the presence of carotenoids (Warhurst and Fewson, 1994). The rhodococci are widespread in nature; members can be found in soil, rocks, ground water, wastes and seawater (Goodfellow and Minniken, 1981).

Many members of the genus *Rhodococcus* possess diverse metabolic capabilities and are found in the microflora of many polluted environments where they play a vital role in natural

degradation, bioremediation and biotransformation (Finnerty, 1992). Biodegradation and biotransformation carried out by *Rhodococcus* is facilitated by the presence of a unique cell envelope and related secretions. Table 1 summarizes some of the capabilities and applications of rhodococci.

Table 1- Some uses and applications of *Rhodococcus*

| Species | Hydrocarbon metabolism ^a | Secretion of surface active lipids ^b | Environmental Applications ^c | Industrial Applications ^d |
|----------------------------|-------------------------------------|-------------------------------------------------|-----------------------------------------|--------------------------------------|
| <i>R. rhodochrous</i> | x | x | | x |
| <i>R. equi</i> | x | | | |
| <i>R. erythropolis</i> | | x | x | |
| <i>R. ruber</i> | | x | | x |
| <i>R. aurantiacus</i> | | x | | |
| <i>Rhodococcus</i> sp P1 | | | x | |
| <i>R. chlorophenolicus</i> | | | x | |

- a- hydrocarbons such as acetylene, acetaldehyde, alcohols, alkanes (German and Knowles, 1988; Sorkhoh et al., 1990, Leahy and Colwell, 1990).
- b- Surface active lipids (glycolipids, steroids, monoglyceride, phosphatidylcholine) and polysaccharide (Philp et al. 2002, Wolfaardt et al. 1994; Urai et al., 2006)
- c- degradation of recalcitrant compounds such as polychlorinated biphenyls (PCB's), s-triazines, sulphonated azo dyes and n-methyl carbamates (Boyle et al., 1992; Heiss et al., 1992; Mulbry, 1994; Behki et al., 1994).
- d- acrylic polymer manufacture (Hughes et al., 1998) production of acrylamide. (Kobayashi and Yamada, 1996), treatment of waste (Aitken, 1993; Ganguly and Pierce, unpublished), production of poly(3-hydroxyalkanoic) acids (Pieper and Steinbuchel, 1992)

Biotransformation of Nitriles

Biotransformation of nitriles is accomplished by hydrolysis reactions catalyzed by nitrile hydratase (NHase), amidase (Kobayashi and Shimazu, 1998). NHase is a soluble metalloenzyme that catalyses conversion of nitriles to amides. Amidase catalyses the formation of carboxylic acid from the corresponding amide.

NHase is a heteromer of equal amounts of α and β subunits, of molecular weight 23 kDa, a non heme iron or a non-corrinoid cobalt occupies the catalytic center (Sugiura et al, 1988; Kobayashi and Shimazu, 1998). Banerjee et al. (2002) suggested two reasons for the association with the metal. One being that metal ions are good catalysts for the hydration of the CN triple bond and that the metal is required for the folding of the enzyme. NHases associated with cobalt have threonine within a critical sequence whereas the ferric NHases have serine. The amino acid sequence of iron and cobalt NHases show significant homology, although there is variation in their conversion capability and substrate specificity (Payne et al., 1997).

Depending on the inducer used and in the presence of cobalt ions selected *Rhodococcus* species produce two NHases (Komeda et al., 1996). These two NHases are called heavy mass (H-NHase) and low mass (L-NHase) NHases depending on the number of subunits present; 4 subunits: 2 of each α and β for L-NHase and 18-20 subunits: 9-10 of each α and β for H-NHase. The H-NHase has a higher affinity for acrylonitrile and is currently employed in the commercial production of acrylamide (Kobayashi et al, 1992). Nagasawa et al. (1988) previously demonstrated that H-NHases have a higher affinity for aliphatic nitriles while

Komeda et al. (1996) subsequently demonstrated that the L-NHases have a higher affinity for aromatic nitriles. The presence of H-NHase and L-NHase enables the organism to use different nitrile compounds as substrates. This increases the substrate range.

Watanabe et al. (1987) examined the effects of culture conditions on NHase activity. Selected nutrients such as meat extract, peptone, and casamino acid corn steep liquor, and yeast extract were evaluated for effects on NHase activity. Yeast extract was shown to be the most favorable. NHase activity can be further enhanced by the addition of inducers such as cyclohexanecarboximide used as an inducer for the *Rhodococcus rhodochrous* J1 strain (Kobayashi et al, 1992). *Rhodococcus* sp DAP 96253 NHase activity is induced remarkably by the presence of cobalt ions in carbohydrate rich media supplemented with urea and selected inducers. Nitriles are not generally used as inducers, however, AN a substrate of the reaction has been shown to induce NHase activity in *Rhodococcus* DAP 96253. A cocktail consisting of ACN (150ppm), AN (150ppm), SN (50ppm), sodium cyanide(10ppm) and crotononitrile (150ppm) are inducers of NHase system (Pierce, US Patent, 1998). The amino acid asparagine (ASN) is similar in structure to acrylonitrile, also induces high levels of this enzyme (Ganguly and Pierce, unpublished).

Stabilization of Nitrile Hydratase

The large scale implementation of rhodococcal cells for the biological treatment of acrylonitrile production wastewater requires the stabilization of NHase. This can be achieved by the immobilization of whole cells or purified enzymes (Bickerstaff, 1997). *Rhodococcus* DAP 96253 was immobilized using calcium alginate or glutaraldehyde and stability of NHase

assessed at 55°C. Glutaraldehyde was superior to calcium alginate in stabilizing NHase (Ganguly and Pierce, unpublished).

Glutaraldehyde ($\text{HCO}-(\text{CH}_2)_3\text{-CHO}$), is one of the most effective protein crosslinking reagents (Russel and Hopwood, 1976). Glutaraldehyde reacts, with free amino groups especially with that of lysine, with sulfhydryl groups, and with phenolic and imadazole rings of amino acids (Habeeb and Hiramoto, 1968). These reactions with proteins results in proteins in the cell becoming closely packed (Jearanaikoon and Abraham-Peskir, 2005).

The use of whole cell rhodococcal glutaraldehyde calalysts is very promising in the detoxification of waste water (Ganguly and Pierce, unpublished) however the immobilization and crosslinking process is harsh on the cell and results in loss of NHase activity. However, despite initial losses in activity, greatest stability (i.e long term) was achieved. Loss of enzyme activity due to immobilization of cells with glutaraldehyde can be due to cross-linking of polypeptide chains of a protein, “this limits the flexibility of a protein and produces stress that if not distributed uniformly along protein chains can lead to the structure of the polypeptide being destroyed,”(Tzanov et al., 2003). Excessive crosslinking with glutaraldehyde can also lead to loss of enzyme activity due to distortion of the three dimensional structure. Costa et al. (2001) studied the effect of increasing glutaraldehyde concentrations from 0.2 to 0.6% v/v on catalase activity at pH 7 at 30°C. The catalase activity decreased with increasing concentration of glutaraldehyde, due to the promotion of cross linking of protein chains with glutaraldehyde.

Improvements in NHase activity and stability, in native cells, can lead to greater activity and stability after immobilization with glutaraldehyde. The cell envelope plays a role, in

enzyme activity and stability, as it controls the type of compounds that enter and leave the cell and impacts the survival of the cell during different environmental conditions such as extremes in temperature and pH. *Rhodococcus* possesses a unique cell envelope, features of this envelope are examined in the next section.

The *Rhodococcus* cell envelope

Taxonomically, members of the genus *Rhodococcus* are placed within the nocardioform actinomycetes (Goodfellow, 1989), and are members of the well characterized suprageneric mycolata taxon. Other members of the mycolata include *Corynebacterium*, *Dietzia*, *Gordonia*, *Mycobacterium*, *Nocardia*, *Skermania* and *Tsukamurella* (Goodfellow, 1992) all of which possess a cell envelope which is of importance in determinative systematics. Table 2 shows the major cell envelope components of taxonomic importance found among mycolata.

Structural components and arrangement

Within the mycolata the cell envelope of the pathogenic *Mycobacteria* such as *M. leprae*, *M. tuberculosis*, *M. avium* and *M. intracellulare* has been studied extensively. The current model of the *Rhodococcus* cell envelope is based upon the mycobacterial cell envelope initially proposed by Minniken (1991) and subsequently revised by Sutcliffe (1997) in relation to *Rhodococcus equi*. While Gram-positive, the mycolata share a characteristic of Gram-negative bacteria, in that they have an outer barrier which is distinct from the cell membrane. An outer permeability barrier is formed as a result of a mono-layer of mycolic acids binding to the cell wall. Evidence of this outer barrier is the existence of cell envelope proteins that possess pore

forming ability (Trias et al., 1992). Freeze fracture analyses indicate that the mycolata have two planes of weakness in their cell envelope in addition to the plasma membrane (Benedetti et al., 1984; Chami et al., 1995) indicating the presence of an outer membrane barrier. Erosion of the cell envelope of mycobacteria and the use of selective lipophilic probes have indicated the presence of lipid domains, providing further evidence of an outer membrane barrier (Ortalo-Magné et al., 1996; Christensen et al., 1999).

Table 2. Characteristics of the cell envelope of mycolata^a

| Characteristics | <i>Corynebacterium</i> | <i>Dietzia</i> | <i>Gordonia</i> | <i>Mycobacterium</i> | <i>Nocardia</i> | <i>Rhodococcus</i> | <i>Skermania</i> | <i>Tsukamurella</i> |
|-------------------------------------------------------------|------------------------|----------------|-----------------|----------------------|-----------------|--------------------|------------------|---------------------|
| Peptidoglycan type ^b | Aly | Aly | Aly | Aly | Aly | Aly | Aly | Aly |
| Acyl group of muramic acid ^c | N-acetylated | N-acetylated | N-glycolated | N-glycolated | N-glycolated | N-glycolated | N-glycolated | N-glycolated |
| Fatty acid types ^d | S, U, T ^e | S, U, T | S, U, T | S, U, T ^f | S, U, T | S, U, T | S, U, T | S, U, T |
| Mycolic acid (MA) Types ^g | Single spot | Single spot | Single spot | Multiple spots | Single spot | Single spot | Single spot | Single spot |
| Number of carbons in MA ^h | 22-38 | 34-38 | 46-66 | 60-90 | 48-60 | 30-54 | 58-64 | 64-78 |
| Number of double bonds | 0-2 | ND | 1-4 | 1-3 | 0-3 | 0-2 | 2-6 | 1-6 |
| Fatty acid esters released on pyrolysis (Number of carbons) | 8-18 | ND | 16-18 | 22-26 | 12-18 | 12-16 | 16-20 | 20-22 |
| Phospholipid Type ⁱ | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |

a- data taken from Goodfellow et al. (1998), Chun et al (1999), Goodfellow and Magee (1997) and Yassin et al. (1997)

b- A, cross-linkage between positions 3 and 4 of adjacent peptide subunits, 1, peptide bridge absent; y, meso-A2p... at position 3 of the tetrapeptide subunits (Schleifer and Kandler, 1972)

c- Acyl group detected using simple glycolate test (Uchida and Aida, 1979)

d- S, Straight chain; U, monounsaturated; T, tuberculostearic acid

e- *Corynebacterium bovis* contains tuberculostearic acid (Lechevalier et al., 1977)

f- *Mycobacterium gordonae* only contains traces of tuberculostearic acid (Minniken et al., 1985)

g- Number of mycolic acids produced from whole organism methanolysates (Minniken et al. 1975, 1984 a, b; Yassin et al., 1997)

h- In mycobacterial mycolic acids, double bonds may be converted to cyclopropane rings; methyl branches and other oxygen functions maybe present (Dobson et al., 1985; Minniken et al., 1984 a, b)

i- Phospholipid types: 1, phosphatidylglycerol (variable) and phosphatidylinositol; 2, phosphatidylethanolamine (Lechevalier et al., 1977)

The rhodococcal outer layer is made up of cell wall linked fatty acids called mycolic acids (Minniken, 1991). The plasma membrane is covalently linked to a mycolic acid, arabinogalactan (AG), peptidoglycan complex. AG is composed of polymers of arabinose and galactose. The galactose residues form a linear galactan polymer which has attached side branches of arabinan (polymer of arabinose). Some muramic acid residues in the peptidoglycan are cross-linked to the galactan polymer in the AG by phosphoryl-N-acetylglucosaminosyl-rhamnosyl linkage units. The arabinan chains are attached to some of the galactose residues linked to the peptidoglycan. The peptidoglycan-arabinogalactan structure forms the cell wall skeleton to which mycolic acids are attached (Crick et al., 2001).

The Sutcliffe model (1997) is depicted in Fig.1. The figure shows the arrangement of the mycolic acids according to their physical and chemical properties. The model of the cell envelope shows the bound mycolic acids attached to arabinogalactan wall polysaccharide through a conserved linkage unit, in a perpendicular orientation. Sutcliffe (1997) suggested that free mycolic acids are intercalated with bound mycolic acids since the free mycolic acids have similar sizes as the bound mycolic acids.

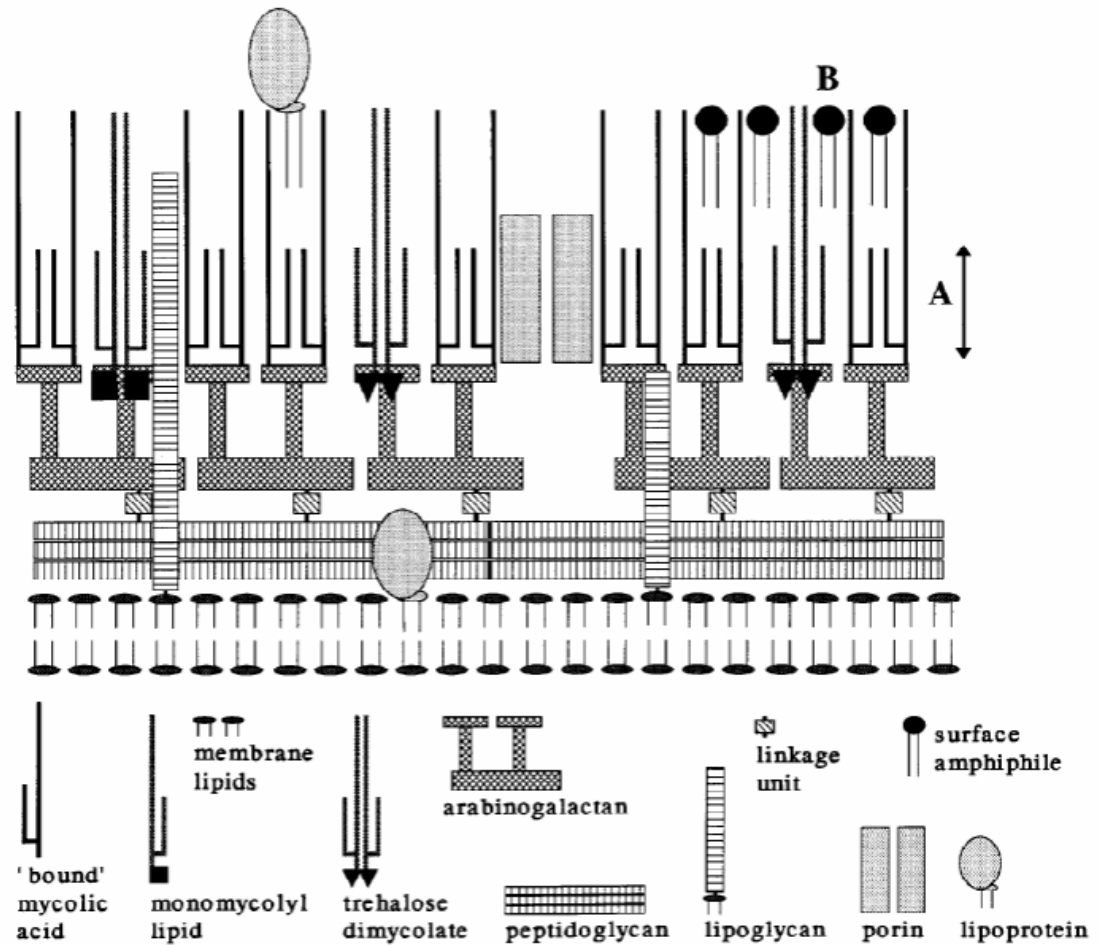


Fig. 1. Cell envelope organization in *Rhodococcus*. (Sutcliffe, 1998).

Mycolic acids – a major component of the *Rhodococcus* cell envelope

Mycolic acids are α -alkyl, β -hydroxy chain fatty acids, that contain a species-dependent saturated α - branch (short arm), consisting of 10-14 carbon atoms and a meromycolate chain (long arm) (Fig 2) that can have varying number of double bonds. Minniken and O'Donnell (1984) suggested that the unsaturated bonds may be located

in the distal regions of the meromycolate chain. There are three structural classes of mycolic acids found in *Mycobacterium tuberculosis*, α , methoxy and keto mycolic acids. Mycolic acids of the α -subclass are the most prevalent, they are cis cis dicyclopropyl fatty acids (Takayama et al., 2005).

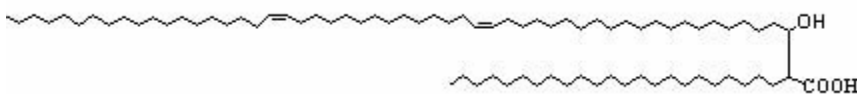


Fig. 2. Generalized structure of a mycolic acid.

Pyrolytic cleavage (300°C) of a mycolic acid, releases an intact fatty acid -the α -alkyl branch (short arm) and an aldehyde (the meroaldehyde) (See Fig. 3).

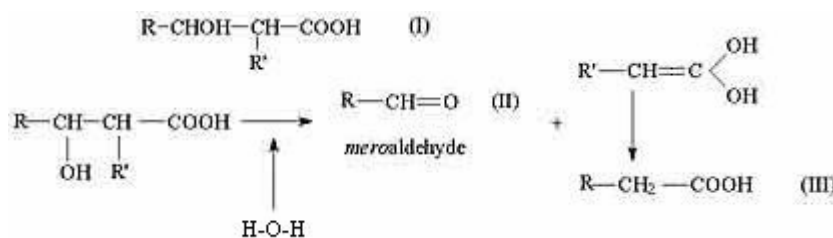


Fig. 3. Pyrolytic cleavage of mycolic acid (I), II) meromycolate main branch or long arm, III) α -alkyl branch (short arm) (Asselineau and Lane lle, 1998).

There are some differences between the arabinans of *Rhodococcus* and *Mycobacterium* that affect the amount of mycolic acids which can be bound to the arabinogalactan skeleton. Besra et al. (1995) showed that *Mycobacterium* arabinan contained a terminal pentarabinosyl branched motif, mycolic acids are esterified to the

termini of arabinan branches. *Mycobacterium* would be able to accommodate four mycolic acids (Fig. 4).

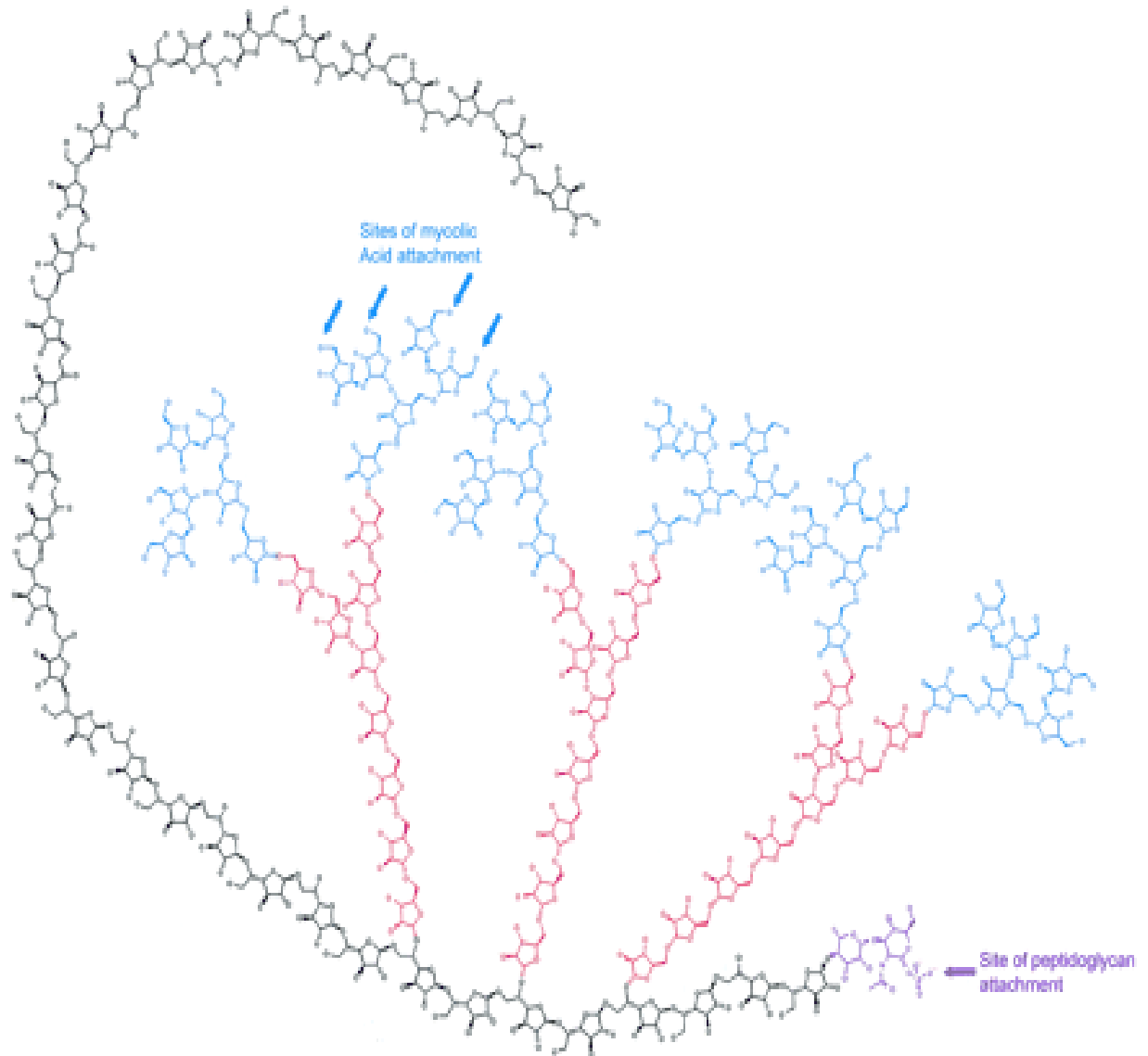


Fig. 4. A typical arabinogalactan molecule from *M. tuberculosis*. The galactan (black) is made up of Galf residues in alternating 1→5, 1→6 linkage. Arabinan (red and blue) is attached to the 5-position of some of the Galf residues, probably near the reducing end of the galactan. The arabinan is composed of Araf residues in 1→5 linkage with

some branching at the 2 and 3 positions. Mycolic acids are attached in clusters on all four of the available five positions (blue arrows) of approximately two-thirds of the terminal hexa-arabinoside motif (blue) by ester linkages. The linker region (purple) is covalently bound to the peptidoglycan via a phosphodiester to the 6-position of a muramate residue. (Crick et al. 2001)

Daffe et al. (1993) showed that the arabinan of *Rhodococcus equi* possessed a branched triarabinosyl motif rather than the branched pentarabinosyl motif present in *Mycobacterium*. Evidence suggests that as a result of the differences in the number of branched arabinosyl terminal motifs, the cell envelope of *Rhodococcus* carries fewer mycolic acids. Hence the cell envelope of *Rhodococcus* is less dense than *Mycobacterium*.

Mycolic acids can either be bound or 'free'. The free mycolic acids are linked to a trehalose (trehalose monomycolates or trehalose dimycolates). The size of the free are thought to be similar to bound mycolates (Tomiyasu and Yano, 1984). Free mycolic acids act as plugs in the outer layer of the cell envelope (Sutcliffe, 1998) forming an outer lipid bilayer. Winder et al. (1967) showed that mycobacteria treated with isoniazid accumulated free trehalose. (Isoniazid is an antibiotic that inhibits mycolic acids biosynthesis. Without mycolic acids trehalose would no longer be able to become incorporated in the outer layer of the cell envelope hence it's accumulation in the cell).

Lipoglycans

The rhodococcal cell envelope also contains lipoglycans (LG). These are macroamphiphilic molecules composed of a polysaccharide attached to lipid, where the lipid anchors the molecule in the cell membrane (Sutcliffe, 1994). Lipoteichoic acids are well studied LGs that are prevalent in Gram-positive bacteria. LGs of *Rhodococcus* have not been intensively researched hence their physiological role and precise function is unknown. Research conducted on LGs from *Mycobacteria*, lipoarabinomannans (LAMs) and lipomannans (LM) show that LAMs and LMs have a membrane anchor based on phosphatidylinositol. The polysaccharide portion of LAMs contains two homopolysaccharides, D- mannan composed of mannopyranose residues and D-arabinan composed of arabinofuranose residues (Nigou et al., 2003).

The location of the LG in *Mycobacteria* and *Rhodococcus* is subject to debate. LG and lipoteichoic acids of other Gram-positive bacteria are anchored to the plasma membrane. Daffe and Draper (1998) showed that LG in mycobacteria was retained in the plasma membrane. However, Brennan and Nikaido (1995) suggested that LG also could interact with the mycolic acid layer. Hence LG also might be localized in the outer bilayer. Despite the location of LG in the cell envelope, LAM-like molecules have been identified in *Rhodococcus ruber* (Besra, unpublished). Flaherty et al (1996) isolated LAM-like and LM-like macromolecules from *Rhodococcus rhodnii* and similar fractions have been found in *R. equi* (Garton et al., unpublished).

Cell envelope proteins

There are many proteins located in the rhodococcal cell envelope. Isolated envelopes from rhodococci have been shown to be composed of approximately 10% protein by weight (Dufrene et al., 1997). The 'pseudoperiplasm' of the mycolata contains many different proteins that are thought to function in the synthesis and assembly of cell envelope components. Proteins may be intimately associated with the outer lipid layer; an example of such a protein in *Rhodococcus* is cholesterol oxidase (Sutcliffe, 1998). Atart et al. (1992) showed that cholesterol oxidase (CO) was localized at a distance up to 80 nm above the cell surface where the CO belonged to a surface layer that had a high carbohydrate content, such as the outer layer made up of arabinose, galactose and trehalose, CO also was located within the cell wall, in the cytoplasmic membrane and in the peripheral cytoplasm.

Porins represent a separate class of rhodococcal envelope proteins in the outer layer to transport hydrophilic molecules through the outer layer (Trias et al., 1998).

Lipoproteins are widely distributed in rhodococcal cell envelopes. *R. equi* incorporates palmitic acid, into at least seven different proteins (Sutcliffe and Prescott, unpublished). Other lipoproteins may be plasma membrane associated. More studies need to be conducted to define the role of these lipoproteins in the cell envelope.

Surface layer proteins

Some members of the mycolata possess a surface layer (S-layer) that is common to many bacteria such as *Bacillus stearothermophilus*, *Lactobacillus helveticus*, *Corynebacterium glutamicum* (Sara and Sleytr, 1994; Lortal et al., 1992, Chami et al., 1995). The S-layer is composed of a crystalline array of proteinaceous subunits. These ordered arrays are due to the presence of a surface secreted protein. S-layers are mostly composed of a single protein or glycoprotein, 40,000-200,000 Daltons. (Sára and Sleytr, 1994).

Freeze fracture electron microscopy of *Corynebacterium glutamicum* has revealed an S-layer composed of two major proteins PS1 and PS2 (Peyret et al., 1993). Puech et al. (2000) reported the presence of mycolyl transferase activity in the PS1 of *C. glutamicum*. Presently the only other member of the mycolata known to have an S-layer is *Mycobacterium bovis* BCG (Lounatmaa and Brander, 1989).

Changes in cell envelope composition

Barry and Mdluli (1996) showed that the regulation of the mycolic acid content in the rhodococcal cell envelope is similar to the regulation of fatty acids present in the plasma membrane. There are different subclasses of mycolic acids and changes made to the structure of mycolic acids can affect the permeability of the cell wall, which can have a profound impact on the organism's susceptibility to antibiotics and other chemotherapeutic agents (Brennan and Nikaido, 1995).

Takayama et al. (2005) commented that the strength of the cell wall of *M. tuberculosis* was related to cyclopropane rings. These rings offered protection from oxidative stress. Another class of mycolic acids, keto-mycolates was examined in *M. tuberculosis*. Deletion of keto-mycolates affected the survival of *M. tuberculosis* in macrophages. Mycolic acids can contribute to the virulence of *Mycobacterium*.

Mycolic acid composition and concentration can be affected by changes in growth phase, culture conditions and growth media. Startton et al. (2003) examined the effects of growth phase on mycolic acid composition and showed that the proportion of saturated mycolic acids and the length of carbon chains changed with the age of the culture.

Increase in growth temperature also leads to changes in mycolic acids. There was an increase in the saturation of mycolic acids in a *Rhodococcus* strain (previously known as *Nocardia rubra*), and increased mean chain length in mycolic acid in *M. phlei* (Tomiyasu et al, 1981; Toriyama et al, 1980). Kremer et al. (2002) showed that growth of *M. thermoresistable* at 55°C led to down regulation of KasA (an enzyme within FAS-11 system used in mycolic acid production). The decrease in the expression of KasA correlated with decreased production of mycolic acids in *M. thermoresistable*. Growth at 55°C resulted in increased levels of unsaturated α -mycolates and methoxymycolates.

Wick et al. (2002) showed that mycolic acid profiles were altered by changing the growth substrate. Sokolovska et al. (2003) also reported that the carbon source influenced mycolic acid composition and cell wall permeability. Growth on hydrophobic substrates

(e.g. alkanes and polycyclic aromatic hydrocarbons) led to a shift in more hydrophobic mycolic acids. Startton et al. (2003) observed significant differences between cells of *Rhodococcus* sp strain 11R grown on glucose or Tween® 80 using the technique selective ion monitoring (SIM) GC-MS. Cells grown on Tween® 80 exhibited less diversity in their mycolic acid composition, they also had carbon lengths of C₃₂-C₄₂ while glucose grown cells had C₃₂-C₄₄. Tween® 80 grown cells had mostly monounsaturated mycolic acids while glucose grown cells had saturated and monounsaturated mycolic acids.

Hashimoto et al (2006) investigated the effect of glutamate overproduction on mycolic acid composition on *Corynebacterium glutamicum*. Glutamate overproduction is induced by biotin limitation and by treatment with detergents or antibiotics. The results showed that glutamate overproduction led to a decreased mycolic acid production with shorter mycolic acids being produced. Korenelli et al. (1990) showed an increase in the glycolipid trehalose dimycolate after *Rhodococcus maris* was grown on hydrophobic substrates as opposed to ethanol substrates.

Trehalose and its role in protein stabilization

Trehalose is a non-reducing disaccharide of D-glucose that is found in many plants, insects, and microorganisms. Trehalose, in solution has been shown to preserve the activity of proteins under stressful conditions such as dehydration, and heat (Colaco et al., 1992; Sola-Penna et al., 1997; Sun and Davidson, 1998; Xie and Timasheff,

1997). Sola-Penna et al. (1997) showed that trehalose was a superior sugar to sucrose, glucose and fructose in protecting enzymes. Xie and Timasheff (1997) showed that trehalose, in solution, stabilized the protein ribonuclease A during exposure to high temperatures. Kaushik and Bhat (2003) by analyzing the thermal stability of five different proteins ribonuclease A, lysozyme, and cytochrome C in the presence of trehalose concluded that trehalose is an exceptional protein stabilizer. Trehalose was predicted to function as a universal protein stabilizer as it was able to stabilize a wide range of proteins used in the study.

There are different opinions on how sugars offer protection to proteins. One such opinion is preferential hydration. This refers to the accumulation of water near the protein (Arakawa, 2002). Polar groups on the surface of proteins are bound to water molecules hence proteins are hydrated in aqueous solution. If this hydration is maintained in the presence of a sugar (cosolute), a difference in the concentration of the sugar develops between the bulk solution and the vicinity of the protein, resulting in preferential hydration where excess water accumulates near the protein. This is which leads to cosolute-induced stabilization (Arakawa, 2002). Timasheff (1992, 1993) explained protein stabilization due to sugars differently. It was shown that when sugar levels increase in bulk water with protein there is competition for available water, this competition leads to less water surrounding the protein. The solvation layer around the protein becomes reduced. The protein becomes more compact and stabilized, being less susceptible to heat and dehydration. Whatever the mechanism involved, trehalose provides exceptional stability to a variety of proteins therefore there is a great demand

for this sugar. It is currently being mass produced from starch (Chaen, 1997) and is being used extensively in the cosmetic, pharmaceutical, medicinal and food industries.

Trehalose can be synthesized from glucose, maltodextrins (polymers of glucose) or maltose. These three routes for trehalose synthesis is present in *Corynebacterium glutamicum* and are shown in Fig.5.

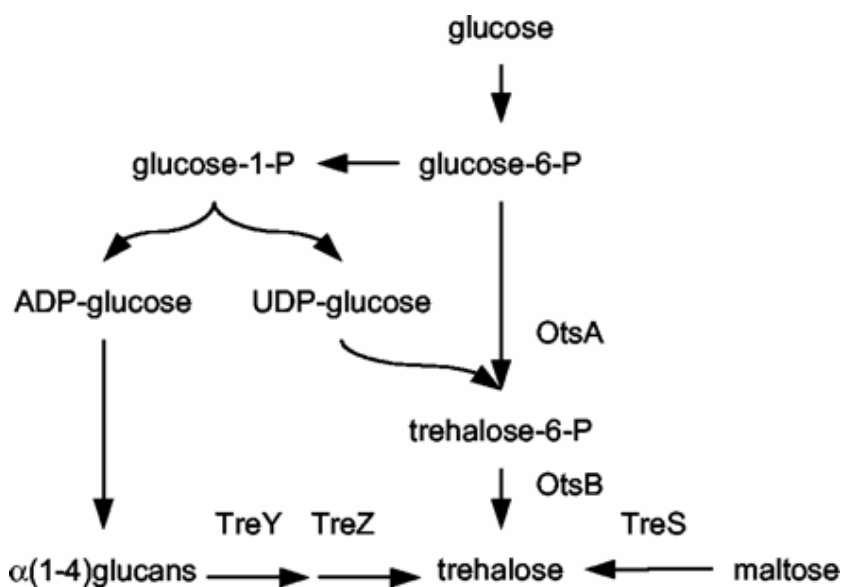


Fig. 5. The three routes for trehalose synthesis in *C. glutamicum* and *M. tuberculosis* (Padilla et al., 2004).

Tzvetkov et al. (2003) analyzed the *Corynebacterium* genome and located genes involved in all three pathways for trehalose synthesis (Fig 6). The genes *otsA*, *otsb*, *treY*, *treZ* and *treS* were knocked out, the cell wall lipid bilayer was affected due to the lack of trehalose production.

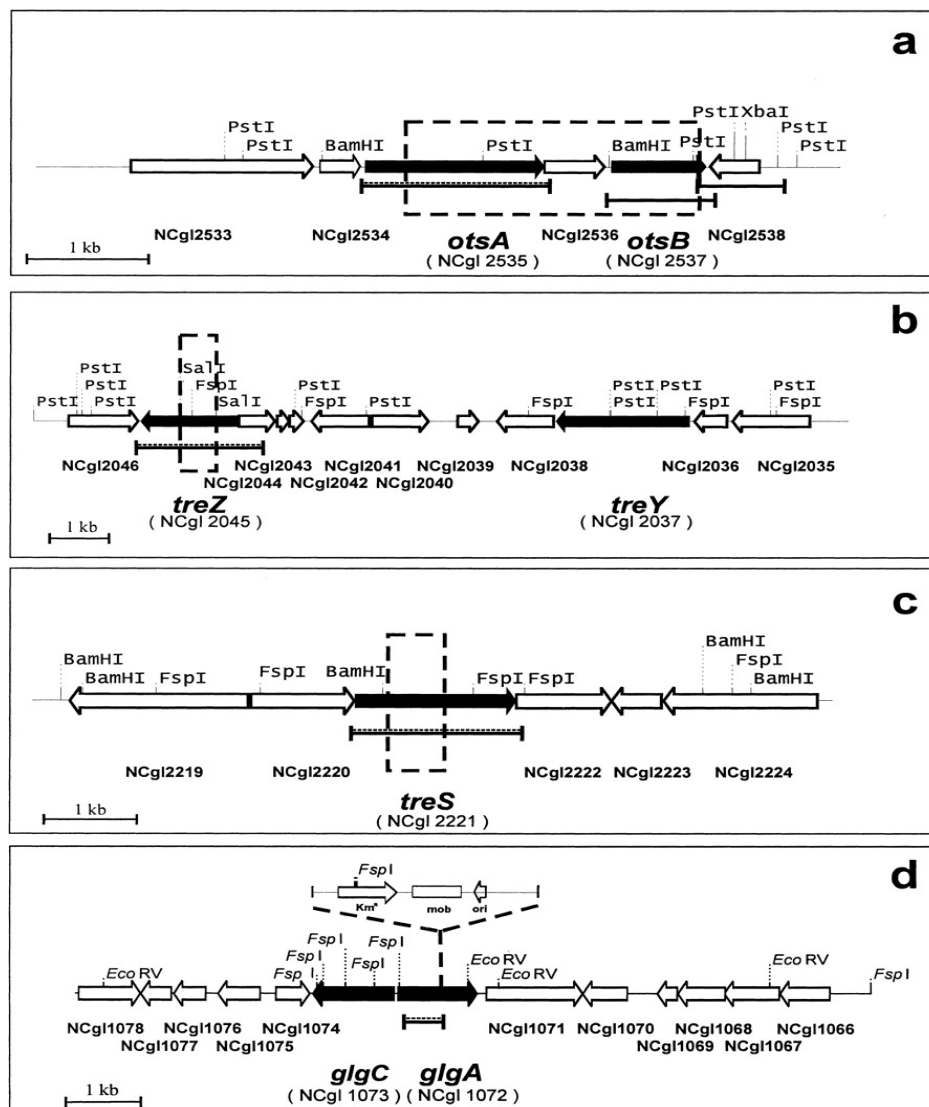


Fig. 5b. Organization of the trehalose (a, b, c) and glycogen (d) biosynthesis genes on the *C. glutamicum* chromosome. The genes directly involved in biosynthesis of trehalose [*otsA/otsB* (a), *treY/treZ* (b) and *treS* (c)] and of glycogen [*glgA/glgC* (d)] are drawn in black (Tzvetkov et al., 2003).

Determination of cell wall permeability

The lipid rich envelope of mycobacteria and other organisms of the mycolata act as a significant barrier. Hydrophilic chemicals and nutrients cannot easily diffuse across the hydrophobic layer and enter the cell through porins. Permeability of the membrane can be influenced by mycolic acid (composition and concentration) and other lipids present in the membrane. Cell wall permeability can be assessed by antibiotic susceptibility. Differences in susceptibilities to an antibiotic may be correlated to changes in the permeability of the cell envelope (Brennan and Nikaido, 1995).

Rationale

NHase from *Rhodococcus* sp DAP 96253 has tremendous potential in becoming one of the most important industrial enzymes. However before the implementation of NHase in industrial processes the enzyme must demonstrate stability for prolonged periods of time with high activity. Previous work showed that NHase from *Rhodococcus* sp DAP 96253 can be stabilized when immobilized with glutaraldehyde. However a significant loss of activity occurs during the preparation of the glutaraldehyde catalyst. This problem can be addressed by using native cells with increased NHase activity and stability. This can lead to a more efficient catalyst which is of tremendous economic value during large scale operation.

Cell envelope components such as mycolic acids and glycolipids can influence the entry of many chemicals such as substrates and immobilizing agents into the cell.

Trehalose, a component of the cell wall, plays a role in the stability of proteins and might have an effect on NHase. This research was based on the following hypotheses:

- 1) NHase activity in native cells can be increased through changes in growth media composition.
- 2) Growth media composition influences NHase stability as it affects trehalose levels inside the cell.
- 3) Changes in growth media composition (such as supplementation of different sugars and inducers for NHase) elicits changes in the cell envelope of *Rhodococcus* sp DAP 96253.

Materials and Methods

Microbiological

Four media listed below were evaluated for their effect on the NHase activity of four rhodococcal species.

1) **Czapek-Dox medium**- D-glucose (20g, MP Biochemicals, Aurora, OH), NaNO₃ (2g, Sigma-Aldrich, St Louis, MO), KH₂ PO₄ (1g, Sigma-Aldrich, St Louis, MO), KCl (1g, Sigma-Aldrich, St. Louis, MO), MgSO₄.7H₂O (0.5g, Fisher Scientific, Fairlawn, NJ), FeSO₄.7H₂O (0.01g, J.T. Baker Chemical Co, Phillipsburg, NJ) in 1 liter and made to a final pH of 4.5.

2) **Glucose Malt Extract (GME)**- D-glucose (40g), malt extract (20g, Becton Dickinson, Sparks, MD), NaNO₃ (2g, Sigma, St. Louis, MO), KCl (1g, Sigma, St. Louis, MO), KH₂ PO₄ (1g, Sigma, St. Louis, MO), MgSO₄.7H₂O (1g, J.T. Baker Chemical Co, Phillipsburg, NJ), FeSO₄.7H₂O (0.02g, Fisher Scientific, Fairlawn, NJ,) in 1 liter.

3) **Yeast Extract Malt Extract (YEMEA) uninduced (UI)**- 4g yeast extract (Becton Dickinson, Sparks, MD), 10g malt extract, 4g glucose in 1L.

4) **YEMEA induced (I)**- 4g yeast extract, 10g malt extract, 4g glucose, cobalt (CoCl₂.6H₂O, 0.201g/l, J.T. Baker Chemical Co, Phillipsburg, NJ,) and urea (7.5g/l, Sigma, St Louis, MO) in 1L..

Plates were made by adding 20g agar (Difco laboratories, Sparks, MD).

The organisms screened were *Rhodococcus* sp DAP 96253, *Rhodococcus* sp. 21090, *Rhodococcus erythropolis* 4177, *Rhodococcus rhodochrous* 33278, *Rhodococcus erythropolis* 47072. All the organisms with exception of the DAP species were obtained from ATCC.

YEMEA and *Rhodococcus* sp DAP 96253 were chosen as the desired media and organism for this research. Glycerol stocks (1ml) of *Rhodococcus* sp DAP 96253 were revived by adding the 1ml to 50ml Nutrient Broth (Difco, Sparks MD). This was grown for 3 days at 30°C. Nutrient agar (Difco, Sparks MD) plates were streaked with the revived organism and grown for 3 days at 30°C. These plates were used to inoculate YEMEA plates. YEMEA plates were incubated at 30°C for about 7 days. To investigate the effect of different sugars on NHase activity fructose, maltose and maltodextrin (all obtained from Sigma, St. Louis, MO) were substituted for glucose in YEMEA media.

Table 3. Media ^a

| Medium | Components (g/L) | | | | | | | | | |
|---------------------------|------------------|----|----|-------------------|---------------------------------|-----|----------------------------------------|--------------------------------------|--------------------------------------|------|
| | G | ME | YE | NaNO ₃ | KH ₂ PO ₄ | KCl | MgSO ₄ 7H ₂ O | FeSO ₄ ·7H ₂ O | CoCl ₂ ·6H ₂ O | Urea |
| CD ^c | 20 | - | - | 2 | 1 | 1 | 0.5 | 0.01 | - | - |
| GME ^d | 40 | 20 | - | 2 | 1 | 1 | 1 | 0.02 | - | - |
| YEMEA ^e -NI | 4 ^b | 10 | 4 | - | - | - | - | - | - | - |
| YEMEA ^e -I | 4 ^b | 10 | 4 | - | - | - | - | - | 0.201 | 7.5 |

CD- Czapek Dox

GME- Glucose malt extract

YEMEA-NI- YEMEA non-induced

YEMEA-I- YEMEA induced

G- glucose

ME- Malt extract

YE- yeast extract

a- 20 g of agar was added to all types of media

b- modified YEMEA media- glucose was replaced with the same amount of fructose, maltose or maltodextrin.

c- Ramachandran and Gottlieb (1963)

d- Pickard (1981)

e- Dietz and Thayer (1980)

Mycobacteria intracellulare ATCC 13950, the control culture for mycolic acid patterns was grown on both Middlebrook agar plates (Difco, Sparks, MD) and Lowenstein Jensen slants (BBL, Sparks, MD) for a week at 37°C as specified by CDC, Standardized method for HPLC identification of *Mycobacteria* and Butler et al., (1986). *Candida albicans* was used as the negative mycolic acid control, and was also grown on Middlebrook agar plates and Lowenstein Jensen slants for a week at 37°C.

Enzyme Assays

NHase was quantified and substrate specificity examined using 1000ppm (v/v) standard solutions of acrylonitrile, propionitrile, butyronitrile and crotononitrile (Aldrich Chemical Company, Milwaukee, WI); individual standard solutions (9 ml) were pipetted into 15 ml centrifuge tubes. A YEMEA plate was scraped to obtain 40 mg of cells (wet wt.), which was suspended into 1 ml of 50mM phosphate buffer (pH 7.2) in an eppendorf tube and combined with 9 ml reaction solution. This suspension was mixed by hand for 2 min then the reaction stopped by reducing the pH by the addition of 200 μ l of 2N sulfuric acid (Fisher Chemical, Fairlawn, NJ), this was mixed for 1 min, then 50 μ l of 8N sodium hydroxide (Fisher Scientific, Fairlawn, NJ) was added to neutralize the acid. Duplicate 1ml samples were placed in separate 1.5 ml Eppendorf tubes and centrifuged (Beckman Microphage Lite, Palo Alto, CA) for 2 min at 13,000 rpm. The supernatant was pipetted into clean eppendorf tubes.

To ensure complete amide conversion to acid and ammonia, commercial amidase (1000 units/ 440 μ l, stored at -20°C) (Sigma-Aldrich Co., St. Louis, MO) was added. A working amidase solution was prepared by making a 1:50 dilution of the original amidase solution, 10 μ l of this working solution was added to each sample for the amide conversion to acid and ammonia, followed by vortexing for 30 sec. These tubes were incubated at 37°C for 30 min.

Ammonia was determined by modification of the method by Fawcett and Scott (1960). 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 0.02N sodium hypochlorite (Clorox, 6.15%), this 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 sec 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. (See Appendix for reagent formulations).

Stability Tests

Cells (50 mg, wet wt) were placed in 1 ml 50mM phosphate buffer and allowed to equilibrate at 10°C, 20°C, 37°C and 55°C for 1hr. An initial NHase activity was determined as described above by reacting the cells with 1000 ppm AN at room temperature. The stability of NHase was assessed by measuring activity at 20 hrs and 45 hrs.

Mycolic acid extraction

Two loopfuls of *Rhodococcus* sp DAP 96253 were scraped from YEMEA plates and 2 ml of saponification reagent added. The tubes were covered, mixed vigorously for 30 sec, the tops wrapped in foil, and then autoclaved for 80 min at 121 psi. Upon cooling, 1.5 ml of acidification reagent as added, to each tube and the tubes content mixed for 30 secs by shaking at room temperature after which the acidified mixture was

extracted 2 times with 1 ml of dichloromethane (DCM) (Sigma-Aldrich, St. Louis, MO). The pooled DCM extracts were dried under nitrogen in a standard heating block (VWR Scientific, Batavia, IL) at 85°C for 15 mins. After drying, 100 µl of potassium bicarbonate reagent was added to the dried extracts and the extracts redried under nitrogen in the heating blocking at 85°C. After redrying, 1ml of DCM and 50 µl of p-bromophenacyl-8 reagent (p-bromphenacyl bromide (0.1mmmol/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 secs then placed in the heating block at 85°C for 25 min. The tubes were cooled and 1 ml of clarification reagent (see appendix) added followed by vortexing for 20 sec. The organic layer was removed with a glass Pasteur pipette and transferred to a clean test tube then evaporated to dryness using the heating block at 85°C under nitrogen for 10 min. The samples were capped tightly and stored at 4°C in the dark [CDC, Standardized method for HPLC identification of *Mycobacteria*; Butler et al., (1986); Durst et al., (1975)].

For HPLC analysis samples were re-solubilized in 500 µl of a solution containing 5 mg of a high molecular weight standard, C110 (Corixa Corporation, Hamilton, MT) in 100 ml DCM. Analysis was performed using a Series 200 HPLC instrument (Perkin Elmer, Shelton, CT) equipped with a UV detector. A Novapak® RP C18 column (Waters, Milford, MA) 3.9 mm by 300 mm, 4 µm, 60A was used. The mobile phase used was methanol and dichloromethane, in a linear gradient of dichloromethane and

methanol from 0-13 min, 0-10%; from 13-17 min, 10-25%; from 17-34 min, 25-75%; from 34-41min, 30-70%; from 41-45 min, 100-0%. The flow rate was 1ml/min and the detector was set at 254nm (Sokolovska et al., 2003). (See Appendix for reagent formulations).

Extraction of total lipids

Cells for lipid extraction were scraped from YEMEA plates then weighed. Approximately 50 mg of cells were suspended in 5 ml solution of chloroform:methanol::2:1 in a 20 ml glass vial. The suspension was incubated for 1 hr at room temperature on automatic shaker (B. Braun, Allentown, PA) set at 120 rpm. Residual bacteria were removed by centrifugation (IEC HN SII Centrifuge Needham Heights, MA, swinging bucket 158 rotor) at 3000 rpm for 15 mins. This was repeated 2 more times using chloroform:methanol::1:2 followed by chloroform: methanol::1:1. The second and third extracts were pooled with the first extract in a 20ml glass vial.

The three pooled extracts were dried using a standard heating block (VWR Scientific, Batavia, IL) under nitrogen at 65°C then resuspended in 100µl of chloroform. Multiple small aliquots (20 µl) were spotted on a TLC plate (Merck, silica gel 60, 10-20cm, 0.25mm thickness) until a total of 100 µl of each sample was spotted. Trehalose dimycolate (Sigma-Aldrich, St. Louis, MO) standard (30 µl total) was also spotted on the plate with the samples

The plates were developed in 90:10:6:1 (chloroform, methanol, acetone and acetic acid). Glycolipids spots were visualized by spraying with a 15% ethanolic solution of 1-naphthol (Aldrich, Milwaukee, WI) followed by heating at 100°C for 3-6 min.

Extraction of cell envelope proteins

Two loopfuls of *Rhodococcus* sp DAP 96253 was scraped from YEMEA plates and added to 5 ml of 50mM phosphate buffer (pH 7.2) and mixed thoroughly by vortexing for 30 sec. A 1.5 ml of this suspension was transferred to a microcentrifuge tube (Eppendorf, 1.5 ml), and centrifuged at 13,000 rpm for 15 min at room temperature using a Beckman Microfuge Lite Centrifuge (Palo Alto, CA) equipped with a F1802B rotor. The supernatant was discarded, and 300 µl of 2% SDS (GE Healthcare Biosciences, Uppsala, Sweden) in 50 mM Tris-HCL (pH 6.8) (Sigma-Aldrich, St Louis, MA) was added to the pellet, followed by vortexing and incubation at room temperature for 5 min.

Other methods of incubation were investigated as follows: using a standard heating block (VWR Scientific, Batavia, IL) at 100°C for 2 and 5 mins or a water bath (100°C) for 2 and 5 mins. Following the incubation, the extracts, were centrifuged at room temperature using a Beckman Microfuge Lite (Palo Alto, CA) equipped with a F1802B rotor for 3 min at 11,400 rpm (Puech et al., 2001). The supernatant was removed and analyzed by 1-D gel electrophoresis. Electrophoresis was carried out using SE 600 Ruby (Amersham Sciences, Piscataway, NJ) at 12.5 mA at 10°C.

Protein bands on a 150 mm x 145 mm gel were visualized using a silver stain according to the method of Switzer et al., (1979). Gels for MADI-TOF/TOF analysis were stained with Coomassie blue (Phastgel™ Blue R, Amersham Biosciences, Piscataway, NJ) then subjected to protein digestion with trypsin (Sigma-Aldrich, St Louis, MO) followed by Zip Tipping (uC18) onto a MALDI TOF/TOF plate. (See Appendix for gel preparation, staining procedures, protein digestion and Zip Tipping).

Analysis of cellular trehalose

Method 1

Cells [50 mg (wet wt.)] were scraped from YEMEA plates into 15ml test tubes containing 5 ml DI water (on ice for 15 min), followed by vortexing and centrifugation at 4°C (IEC HN SII Centrifuge, Needham Heights, MA, swinging bucket 158 rotor) at 3000 rpm; The supernatant was discarded and 200 µl of 500mM trichloroacetic acid (TCA) (Sigma, St. Louis, MO) was added to the pellet and incubated for 1 hr at room temperature with moderate shaking using an automatic shaker (B. Braun, Allentown, PA). A second extraction was performed as mentioned above (Lillie and Pringle, 1980). The extracts were pooled and analyzed for their trehalose content by the anthrone reaction by a modification of the method by Spiro (1966). In preparation for the anthrone reaction, the extracts were diluted (1:10) in glass screw cap test tubes and mixed by vortexing; 5 ml of anthrone reagent (See appendix) was added. A water bath was heated to 100°C and test tubes added to the previously heated water bath at 100°C,

the tubes were incubated for 3 min, cooled and 1ml dispensed in cuvettes to read the absorbance at 625nm using a Turner SP 830 spectrophotometer (Barnstead, Dubuque, IO). Prior to this a glucose (10µg-100µg/ml glucose in DI water) standard curve was prepared.

Method 2

Cellular trehalose was analyzed by adding 100 mg of cells (wet wt.) to 15 ml 10% aqueous TCA and incubating the suspension at room temperature on an automatic shaker (B. Braun, Allentown, PA) set at 120 rpm for 24 hrs. The cells were removed by centrifugation (IEC HN SII Centrifuge, Needham Heights, MA, swinging bucket 158 rotor)) and the supernatant extracted with 30 ml of diethyl ether. The aqueous layer was dried under nitrogen at 65-70°C using a standard heating block (VWR Scientific, Batavia, IL) followed by the addition of 1ml of Tri- Sil A[®] reagent (Pierce, Rockford, IL) (Tri Sil A[®] reagent contains hexamethyldisiloxane (HMDS), trimethylchlorosilane (TMCS) and pure pyridine in a 3:1:9 ratio) followed by vortexing for 30 sec and drying under nitrogen using standard heating block at 65-70°C. Tri Sil A (70 µl) was added to the dried sample, and transferred to a glass vial, a 5 ul aliquot was analyzed by splitless injection using a Perkin Elmer Autosystem XL Gas Chromatograph (Perkin Elmer, Shelton, CT) equipped with an FID detector and an OV1701 capillary column (Ohio Valley, Marietta, OH; 14% cyanopropylphenyl methypolysiloxane, 30m x 0.25mm ID, 0.25µm). Helium was used as a carrier gas, the inlet and detector temperatures were

300°C. The temperature program employed was a 100°C for 2 min, followed by a gradient of 17.5°/min for 10 min, and a final hold at 275°C for 7 min based upon the methods outlined by Elbein and Mitchell (1973) and Caprioli et al. (2004).

Analysis of bound trehalose

Cells (100 mg, wet wt) were scrapped from the plates and extracted overnight with 15 ml of chloroform-methanol (2:1). The precipitate was removed by filtration using glass wool and washed with chloroform:methanol:: 2:1. The filtrate and wash were combined and evaporated to dryness using a standard heating block (VWR Scientific, Batavia, IL) at 65°C. The residue was suspended in 5 ml methanol and treated with 5 ml 1N sodium methoxide for 4 hrs at 37°C, DI water (5 ml) was added to destroy the sodium methoxide and the solution neutralized with 5 ml of 1N acetic acid. The solution was concentrated to dryness under nitrogen using a standard heating block at 65°C and the dried residue resuspended in 1ml water. The resuspended residue was extracted with 1 ml chloroform to remove lipids. The aqueous layer was dried under nitrogen at 65°C using a standard heating block (VWR Scientific, Batavia, IL) and the trehalose content analyzed by anthrone reaction and by gas chromatography as outlined above.

Determining the permeability of the cell envelope by measuring susceptibility of *Rhodococcus* sp DAP 96253 to antibiotics

The susceptibility of *Rhodococcus* sp DAP 96253 to Erythromycin and Rifampin was determined as an indicator for permeability of the cell envelope (Brennan and Nikaido, 1995). Susceptibility was determined by Etest[®], this is a technique that directly quantifies antimicrobial susceptibility in discrete minimum inhibitory concentrations (MIC) values. Etest[®] was performed using Etest[®] strips (AB Biodisk, Piscataway, NJ) that consisted of an inert plastic strip with a MIC scale ($\mu\text{g/ml}$) on one side and an immobilized exponential gradient of antibiotic on the other side. The gradient covers a continuous concentration range across 15 two fold dilutions of a conventional MIC method. The Etest[®] strip was applied to the inoculated agar surface. After one week bacterial growth became visible and a symmetrical inhibition ellipse centered along the strip was observed. The MIC value was read from the scale where the ellipse edge intersected the strip.

Rhodococcus sp DAP 96253 was grown on nutrient agar plates for 3 days, the cells were scrapped and suspended into 5ml 50mM PBS. YEMEA (un-induced and induced with different sugars) spread plates were prepared and dried. The Etest[®] strips were added to the plates and incubated at 30°C for a week. Antibiotic discs (Sensi-Disc, Becton Dickinson and Company, Sparks, MD) of Vancomycin (30 μg), Ampicillin (10 μg) and Tetracycline (30 μg) were added to inoculated YEMEA plates, the plates were incubated at 30°C for a week and the zone of inhibition measured.

Electron Microscopy

Rhodococcus sp DAP 96253 was scraped from plates and placed between thin copper holders and quenched in liquid propane. The frozen samples were fractured at -125°C in a vacuum by removing the upper plate with a liquid nitrogen- cooled knife in a RMC-RFD 9010 (Baltech Institute, Tuscon, AZ). The fractured samples were etched at -100°C for 2mins at 1.3×10^{-5} Pa and then a replica made with platinum-carbon backed with 20nm carbon. The replica was cleaned in sodium hypochlorite for 2hrs then washed with distilled water (Puech et al., 2001) and observed with Leo 906e Transmission Electron Microscope (Carl Zeis SMT, Peabody, MA).

Results

Propagation and induction of enzymes on different media

Several solid media were used to cultivate selected species of *Rhodococcus* to evaluate the effect on NHase activity, to the substrate acrylonitrile (AN). The media evaluated included Czapek-Dox (pH 4.5), glucose malt extract agar (GME), and yeast extract malt extract agar (YEMEA) without cobalt and urea and with cobalt and urea inducers of NHase. The different species evaluated were *Rhodococcus* sp DAP 96253, *Rhodococcus* sp. 21090, *Rhodococcus erythropolis* 4277, *Rhodococcus rhodochrous* 33278, *Rhodococcus erythropolis* 47072. Table 4 shows that three of the organisms did not grow on Czapek –Dox media. *Rhodococcus* sp DAP 96253 had the highest NHase activity on uninduced YEMEA supplemented with glucose (40 units/mg cdw) and induced YEMEA supplemented with glucose (104 units/mg cdw). NHase activity for *Rhodococcus* sp DAP 96253 was low (11 units/mg cdw) both on Czapek-Dox and GME. *Rhodococcus erythropolis* 4277 showed the second highest NHase activity of 34 units/mg cdw on induced YEMEA. Growth on YEMEA media led to the highest NHase production with *Rhodococcus* sp DAP 96253, but growth on the other types of media did not produce such dramatic effects. Based upon this initial screening *Rhodococcus* sp DAP 96253 was the best candidate for NHase production among the species examined and that solid YEMEA was the best media to cultivate the organism, and *Rhodococcus* sp DAP 96253 was selected as the benchmark microorganism for this dissertation.

Table 4- NHase activities (units/mg cdw) of different rhodococcal species grown on different types of media.

| Organism | NHase activity (unit/mg cdw) on different media | | | |
|------------------------------------|-------------------------------------------------|-----|---------------|--------------|
| | Czapek Dox | GME | YEMEA (NI) | YEMEA (I) |
| <i>Rhodococcus</i> sp DAP 96253 | 11 | 11 | 40 | 104 |
| <i>R. rhodochrous</i> 33278 | no growth | 14 | 8 | 28 |
| <i>R. erythropolis</i> 47072 | no growth | 10 | 16 | 29 |
| <i>R. erythropolis</i> 4277 | 7 | 8 | 12 | 34 |
| <i>Rhodococcus</i> sp. 21090 | no growth | 13 | 9 | 15 |

NI-non induced, I – induced, unit- 1uM of acrylonitrile converted to 1 uM acrylamide in 1min, pH 7.2 at 30°C. Non induced YEMEA was supplemented with glucose (4g/L) and induced YEMEA was supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L).

Effect media composition on NHase activity and substrate specificity

Rhodococcus sp DAP 96253 was grown on solid YEMEA supplemented with different carbohydrates (glucose, fructose, maltose, maltodextrin) with and without inducers [cobalt (50mg/L) and urea (7.5g/L)] and the NHase activity against selected nitriles was determined (Table 5, 6). Substrates used were acrylonitrile (AN), propionitrile (PN), butyronitrile (BN), and crotonitrile (CrN).

NHase activities to BN and CrN were significantly lower than the NHase activity to AN and PN, with glucose grown cells having the lowest activity to BN and CrN. Un-induced cells that were grown in the presence of maltodextrin had the highest NHase activity (against AN and PN) (Table 5). Induced cells grown on maltose and maltodextrin had the highest NHase activity to PN out of all the substrates examined (Table 6). Supplementation of maltodextrin in the YEMEA produced cells that had the highest NHase activity to all the substrates except for CrN. Supplementation of maltose, produced cells with the second highest NHase activity to AN, PN and BN.

Glucose supplementation produced cells with the lowest NHase activity to AN, PN and BN. The supplementation of various sugars in YEMEA does have an impact on NHase activity. Table 7 and 8 provides the statistical evaluation of the data using AN and PN as substrates. P-values higher than 0.05 were not statistically significant. NHase activities of un-induced cells grown on YEMEA supplemented with fructose or maltodextrin produced cells whose NHase activities were statistically different from the NHase activity of glucose grown cells. Cells induced and supplemented with fructose, maltose or maltodextrin, all had NHase activities that were shown to be statistically different from the NHase activity of cells grown on inducing media supplemented with glucose (p-values 0.001, 0.018, 0.012 for fructose, maltose and maltodextrin induced cells respectively, Table 7). Glucose, maltose and maltodextrin induced cells had NHase activities that were statistically different from the NHase activity of cells grown on inducing YEMEA supplemented with fructose. NHase activities of cells grown on YEMEA with cobalt and urea, supplemented with maltodextrin were also statistically different from cells grown on YEMEA supplemented with maltose, cobalt and urea. Similar trends were observed when the preferred substrate PN was used, with exception of induced fructose grown cells whose NHase activity was statistically insignificant compared to induced glucose grown cells (Table 8). These results showed that supplementation of different carbohydrates can affect the substrate specificity of NHase and lead to statistically significant differences in activity.

Table 5. NHase activity (units/mg cdw) against selected nitriles for *Rhodococcus* sp DAP 96253 grown on YEMEA without inducers and with supplementation of different sugars

| Substrate | Glucose | Fructose | Maltose | Maltodextrin |
|-----------|---------|----------|---------|--------------|
| AN | 32 | 54 | 60 | 65 |
| PN | 27 | 58 | 69 | 75 |
| BN | 4 | 7 | 12 | 10 |
| CrN | 3 | 7 | 11 | 12 |

YEMEA was supplemented with different sugars such as glucose, fructose, maltose and maltodextrin at 4g/L

unit- 1 μ M of acrylonitrile converted to 1 μ M acrylamide in 1min, pH 7.2 at 30°C

AN- Acrylonitrile, PN-Propionitrile, BN-Butyronitrile, CrN- Crotononitrile.

Table 6. NHase activity (units/mg cdw) against selected nitriles for *Rhodococcus* sp 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 |

unit- 1 μ M of acrylonitrile converted to 1 μ M of acrylamide in 1min, pH 7.2 at 30°C

YEMEA was supplemented with different sugars such as glucose, fructose, maltose, maltodextrin at 4g/L and inducers cobalt (50mg/L) and urea (7.5g/L)

AN-Acrylonitrile, PN-Propionitrile, BN-Butyronitrile, CrN- Crotononitrile

Table 7. Statistical evaluation of NHase activities (units/mg cdw) of *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with different sugars and inducers against acrylonitrile.

| Media | Statistical Evaluation | |
|---------------------------|------------------------|------------|
| | P value | Stan. Dev. |
| F (NI) ^a | 0.04 | 11.27 |
| M(NI) ^a | 0.07 | 11.31 |
| MD(NI) ^a | 0.024 | 14.43 |
| F, Co, U (I) ^b | 0.001 | 11.01 |
| M, Co, U (I) ^b | 0.018 | 23.87 |
| MD, Co, U(I) ^b | 0.012 | 33.55 |
| M, Co, U (I) ^c | 0.024 | 13.60 |
| MD, Co, U(I) ^c | 0.010 | 23.11 |
| MD, Co, U(I) ^d | 0.002 | 12.91 |

F- YEMEA supplemented with fructose (4g/L)

M- YEMEA supplemented with maltose (4g/L)

MD- YEMEA supplemented with maltodextrin (4g/L)

G, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

F, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

M, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

MD, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

NI- non induced

I- induced

a- NHase activities compared with NHase activity of *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with glucose (4g/L)

b- NHase activities compared with NHase activity of *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L).

c- NHase activities compared with NHase activity of *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with fructose (4g/L), cobalt (50mg/L) and urea (7.5g/L).

d- NHase activities compared with NHase activity of *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with maltose (4g/L), cobalt (50mg/L) and urea (7.5g/L).

unit- 1 μ M of acrylonitrile converted to 1 μ M acrylamide in 1min, pH 7.2 at 30°C

Stan. Dev. - Standard Deviation

Table 8. Statistical evaluation of NHase activities (units/mg cdw) of *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with different sugars and inducers against propionitrile.

| Media | Statistical Evaluation | |
|---------------------------|------------------------|------------|
| | P value | Stan. Dev. |
| F, Co, U (I) ^a | 0.07 | 11.82 |
| M, Co, U (I) ^a | 0.007 | 38.24 |
| MD, Co, U(I) ^a | 0.004 | 43.83 |
| M, Co, U (I) ^b | 0.010 | 31.04 |
| MD, Co, U(I) ^b | 0.003 | 36.49 |
| MD, Co, U(I) ^c | 0.030 | 9.70 |

G, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

F, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

M, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

MD, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

I- induced

a- NHase activities compared with NHase activity of *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L).

b- NHase activities compared with NHase activity of *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with fructose (4g/L), cobalt (50mg/L) and urea (7.5g/L).

c- NHase activities compared with NHase activity of *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with maltose (4g/L), cobalt (50mg/L) and urea (7.5g/L).

unit- 1µM of acrylonitrile converted to 1 µM acrylamide in 1min, pH 7.2 at 30°C

Stan. Dev. - Standard Deviation

Effect of temperature on NHase activity

AN specific NHase activity of *Rhodococcus* sp DAP 96253 was measured after incubation at different temperatures (10°C, 23°C, 37°C, 55°C) (Table 9). The cells were grown on YEMEA supplemented with different sugars and inducers. NHase activity was highest at all temperatures when cells were grown on YEMEA supplemented with maltose except for 10°C where maltodextrin supplementation produced the highest activity. Cells grown on media supplemented with glucose, cobalt and urea had the lowest NHase activities at all the temperatures tested. Increasing the temperature for cells grown on YEMEA supplemented with fructose did not increase NHase activity, as observed with cells grown on media supplemented with glucose or maltose, fructose grown cells worked the best at 23°C. This showed that supplementations of different carbohydrates in growth media can facilitate reactions at certain temperatures.

Table 9. Effect of temperature on NHase activity (units/mg cdw) of *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with different sugars and inducers.

| Supplement | NHase activity (units/mg cdw) at different temperatures | | | |
|------------|---------------------------------------------------------|------|------|------|
| | 10°C | 23°C | 37°C | 55°C |
| G, Co, U | 72 | 95 | 100 | 105 |
| F, Co, U | 102 | 140 | 117 | 123 |
| M, Co, U | 123 | 143 | 150 | 157 |
| MD, Co, U | 142 | 120 | 128 | 132 |

G, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

F, Co, U- YEMEA supplemented with fructose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

M, Co, U- YEMEA supplemented with maltose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

MD, Co, U- YEMEA supplemented with maltodextrin (4g/L), cobalt (50mg/L) and urea (7.5g/L)
unit- 1µM of acrylonitrile converted to 1µM acrylamide in 1 min, pH 7.2, at 30°C

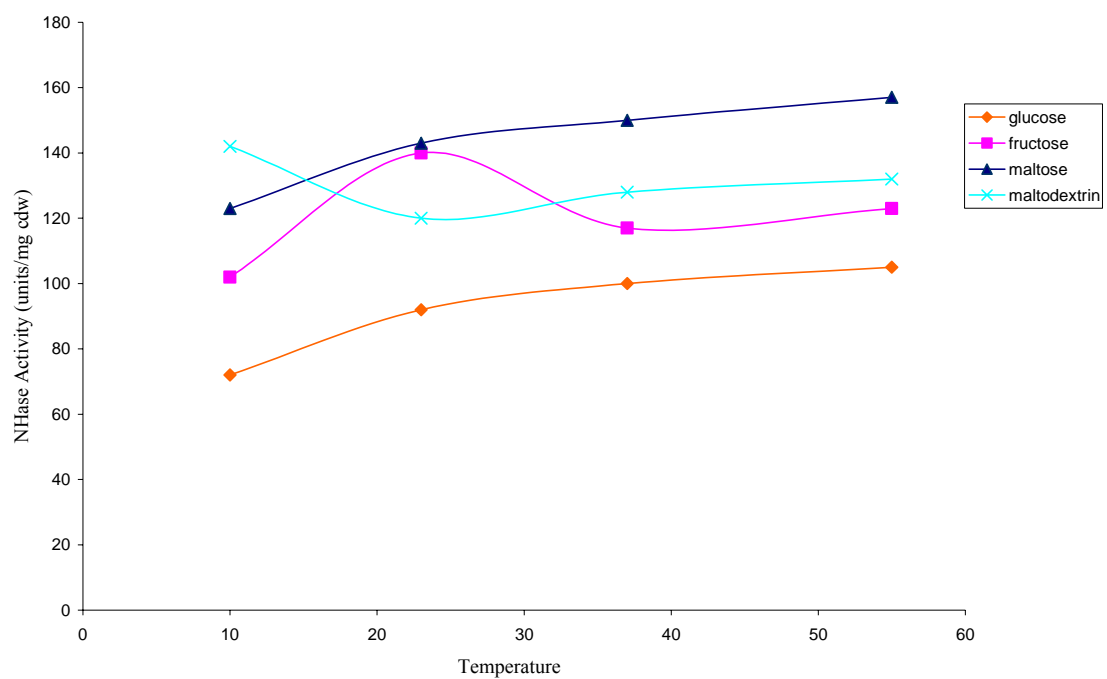


Fig. 6- Effect of temperature (10°C, 23°C, 37°C, 55°C) on NHase activity. *Rhodococcus* sp DAP 96253 was grown on YEMEA supplemented with different glucose (4g/L), fructose (4g/L), maltose (4g/L), maltodextrin (4g/L), cobalt (50mg/L) urea (7.5g/L) and ASN (1g/L) were added as inducers.

NHase stability

The stability of NHase in *Rhodococcus* sp DAP 96253 cells was assessed at 10°C and at 55°C after growth on YEMEA supplemented with different sugars and inducers. Table 10 shows NHase stability over a 40 hour period at 10°C. Cells grown on YEMEA supplemented with fructose cobalt and urea lost the greatest percentage of activity (44%), while cells grown on YEMEA supplemented with maltose or maltodextrin retained the most activity with only a 16% loss. When cells were grown on maltose or maltodextrin supplemented YEMEA the NHase stability was statistically different from the NHase stability of the glucose grown cells, p values were well below 0.05 (p values of 0.006 and 0.005 for maltose and maltodextrin grown cells respectively). Therefore growth of *Rhodococcus* sp DAP 96253 on YEMEA supplemented with different sugars affects the stability of the enzyme at low temperatures.

Table 10. NHase stability of *Rhodococcus* sp DAP 96253 at 10°C after growth on YEMEA supplemented with different sugars and inducers

| Supplement | NHase activity at 10 °C at different time periods (hrs) | | | | % of NHase activity lost | Statistical evaluation ^b | |
|------------|---------------------------------------------------------|-----|-----|-----|--------------------------|-------------------------------------|-------|
| | 0 | 4 | 20 | 30 | | p value | S. D. |
| G, Co, U | 72 | 79 | 80 | 53 | 26 | | |
| F, Co, U | 102 | 70 | 61 | 57 | 44 | 0.234 | 6.344 |
| M, Co, U | 123 | 123 | 117 | 103 | 16 | 0.006 | 28.75 |
| MD, Co, U | 142 | 140 | 136 | 120 | 16 | 0.005 | 38.52 |

G, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

F, Co, U- YEMEA supplemented with fructose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

M, Co, U- YEMEA supplemented with maltose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

MD, Co, U- YEMEA supplemented with maltodextrin (4g/L), cobalt (50mg/L) and urea (7.5g/L)

unit- 1µM of acrylonitrile converted to 1 µM acrylamide in 1 min, pH 7.2, at 30°C

b- All data was compared with NHase activity of cells grown on YEMEA with glucose cobalt and urea after 40 hrs. at 10 °C.

S. D. – Standard deviation

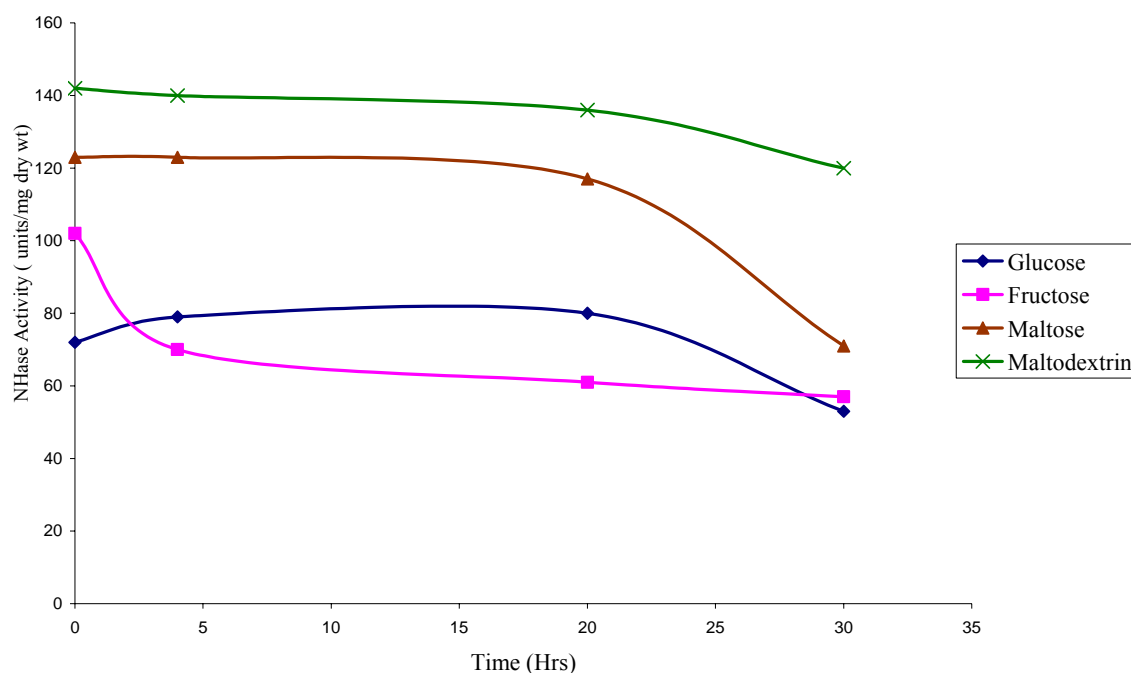


Fig 7. NHase stability at 10°C. *Rhodococcus* sp DAP 96253 was grown on YEMEA supplemented with glucose (4g/L), fructose(4g/L), maltose (4g/L), maltodextrin (4g/L) and inducers cobalt (50mg/L) and urea (7.5g/L).

NHase stability at 55°C was also influenced by changes in media composition.

Additional media formulations were tested at 55°C such as YEMEA with the various sugar supplementations, cobalt, urea and ASN. ASN is a non toxic inducer of NHase and its impact on stability of the native cell was assessed. Trehalose was added to media as a stabilizer for NHase. The effect of this sugar on the stability of NHase was examined.

Table 11 and accompanying Fig 7a and 7b show the NHase activities over a 45 hour period after growth on YEMEA with different supplementations. Cells grown on YEMEA supplemented with glucose, cobalt, urea and ASN lost the greatest percentage

of activity (98%) followed by cells grown on YEMEA supplemented with fructose, cobalt and urea (90%). The addition of trehalose to induced YEMEA supplemented with different sugars resulted in an increased stability of NHase for example cells grown on YEMEA supplemented with fructose, cobalt and urea lost 90% of NHase activity within 45 hrs at 55°C, but cells grown in the presence of trehalose lost only 68% of NHase activity. Cells grown on YEMEA supplemented with maltose, cobalt, urea and trehalose lost the least activity after 45 hrs at 55°C, retaining 41% of NHase activity. Cells grown on YEMEA supplemented with maltose, cobalt and urea lost 55% of their NHase activity after 45 hrs, this was the lowest percentage of NHase activity lost when cells were not grown on media supplemented with trehalose.

Differences observed in NHase stability after growth on YEMEA supplemented with different sugars and inducers were statistically significant. NHase activities for cells grown on YEMEA induced for NHase and supplemented with fructose, maltose or maltodextrin were statistically different from the NHase activities of cells grown on YEMEA induced with cobalt and urea with glucose supplementation, at the various time intervals as indicated by the p values in Table 11. Fig. 7-9 shows the NHase stability graphs for *Rhodococcus* sp DAP 96253 grown on YEMEA with various supplementations and inducers. Table 12 shows that the addition of trehalose to YEMEA supplemented with glucose or fructose made a statistical difference in NHase stability (p-value of 0.007 for glucose supplemented YEMEA and p-value of 0.037 for fructose supplemented YEMEA). The statistical evaluations revealed that addition of

trehalose to YEMEA already supplemented with maltose or maltodextrin did not result in any statistically significant difference in NHase stability.

Table 11. NHase stability ^a at 55°C after growth on YEMEA supplemented with different sugars and inducers.

| Supplements in YEMEA | NHase activity (units/mg cdw) at different time periods (hrs) | | | % of NHase activity lost | Statistical evaluation ^b | |
|----------------------|----------------------------------------------------------------|-----|----|--------------------------|-------------------------------------|-------|
| | 0 | 20 | 45 | | p-value | S. D. |
| G, Co, U | 101 | 32 | 19 | 81 | | |
| F, Co, U | 120 | 22 | 12 | 90 | 0.035 | 4.14 |
| M, Co, U | 133 | 82 | 60 | 55 | 0.030 | 18.71 |
| MD, Co, U | 140 | 105 | 31 | 77 | 0.085 | 13.73 |
| G, Co, U, ASN | 100 | 21 | 12 | 98 | 0.026 | 3.90 |
| F, Co, U, ASN | 84 | 44 | 15 | 82 | 0.014 | 3.87 |
| M, Co, U, ASN | 118 | 31 | 22 | 81 | 0.173 | 4.885 |
| MD, Co, U, ASN | 85 | 40 | 30 | 65 | 0.022 | 5.202 |
| G, Co, U, Tre | 96 | 80 | 38 | 60 | 0.037 | 9.48 |
| F, Co, U, Tre | 101 | 74 | 32 | 68 | 0.002 | 6.89 |
| M, Co, U, Tre | 110 | 85 | 65 | 41 | 0.004 | 22.19 |
| MD, Co, U, Tre | 112 | 90 | 52 | 54 | 0.003 | 17.81 |

G, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

F, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

M, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

MD, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

G, Co, U, ASN- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L), urea (7.5g/L) and asparagine (1g/L)

F, Co, U, ASN- YEMEA supplemented with fructose (4g/L), cobalt (50mg/L), urea (7.5g/L) and asparagine (1g/L)

M, Co, U, ASN- YEMEA supplemented with maltose (4g/L), cobalt (50mg/L), urea (7.5g/L) and asparagine (1g/L)

MD, Co, U, ASN- YEMEA supplemented with maltodextrin (4g/L), cobalt (50mg/L), urea (7.5g/L) and asparagine (1g/L)

G, Co, U, Tre- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L), urea (7.5g/L) and trehalose (4g/L)

F, Co, U, Tre- YEMEA supplemented with fructose (4g/L), cobalt (50mg/L), urea (7.5g/L) and trehalose (4g/L)

M, Co, U, Tre- YEMEA supplemented with maltose (4g/L), cobalt (50mg/L), urea (7.5g/L) and trehalose (4g/L)

MD, Co, U, Tre- YEMEA supplemented with maltodextrin (4g/L), cobalt (50mg/L), urea (7.5g/L) and trehalose (4g/L)

a- stability was determined by measuring NHase activity (units/mg cdw) at time intervals

b- All data was compared with the NHase activity of cells grown on YEMEA with glucose cobalt and urea after 45 hrs. incubation at 10 °C.

S. D. – Standard deviation

Table 12. Statistical evaluation of the stabilizing effect of trehalose when added to YEMEA supplemented with different sugars.

| Media | P value ^a | S. D. ^a |
|----------------|----------------------|--------------------|
| G, Co, U, Tre | 0.007 | 10.12 |
| F, Co, U, Tre | 0.037 | 9.48 |
| M, Co, U, Tre | 0.21 | 5.95 |
| MD, Co, U, Tre | 0.20 | 8.19 |

G, Co, U, Tre- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L), urea (7.5g/L) and trehalose (4g/L)

F, Co, U, Tre- YEMEA supplemented with fructose (4g/L), cobalt (50mg/L), urea (7.5g/L) and trehalose (4g/L)

M, Co, U, Tre- YEMEA supplemented with maltose (4g/L), cobalt (50mg/L), urea (7.5g/L) and trehalose (4g/L)

MD, Co, U, Tre- YEMEA supplemented with maltodextrin (4g/L), cobalt (50mg/L), urea (7.5g/L) and trehalose (4g/L)

a- data compared with NHase activity obtained from cells grown on the corresponding media without trehalose added.

S. D. standard deviation.

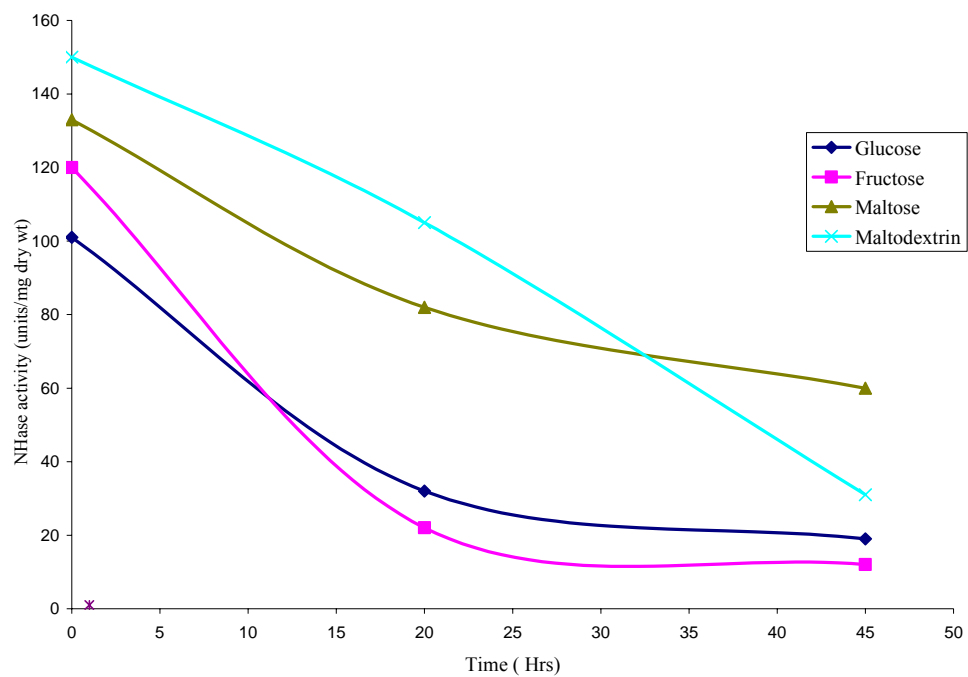


Fig. 7a. NHase stability at 55 °C. *Rhodococcus* sp DAP 96253 was grown on YEMEA supplemented with different glucose (4g/L), fructose (4g/L), maltose (4g/L), maltodextrin (4g/L), and inducers cobalt (50mg/L) and urea (7.5g/L).

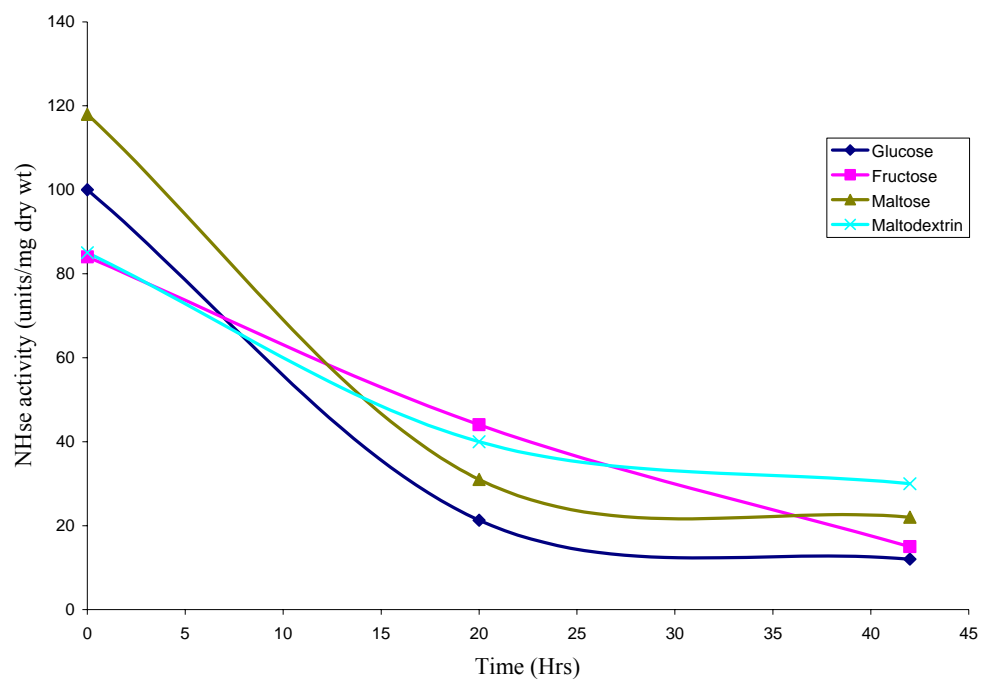


Fig 7b. NHase stability at 55 °C of cells grown on media supplemented with ASN. *Rhodococcus* sp DAP 96253 was grown on YEMEA supplemented with different glucose (4g/L), fructose (4g/L), maltose (4g/L), maltodextrin (4g/L), cobalt (50mg/L) urea (7.5g/L) and ASN (1g/L) were added as inducers.

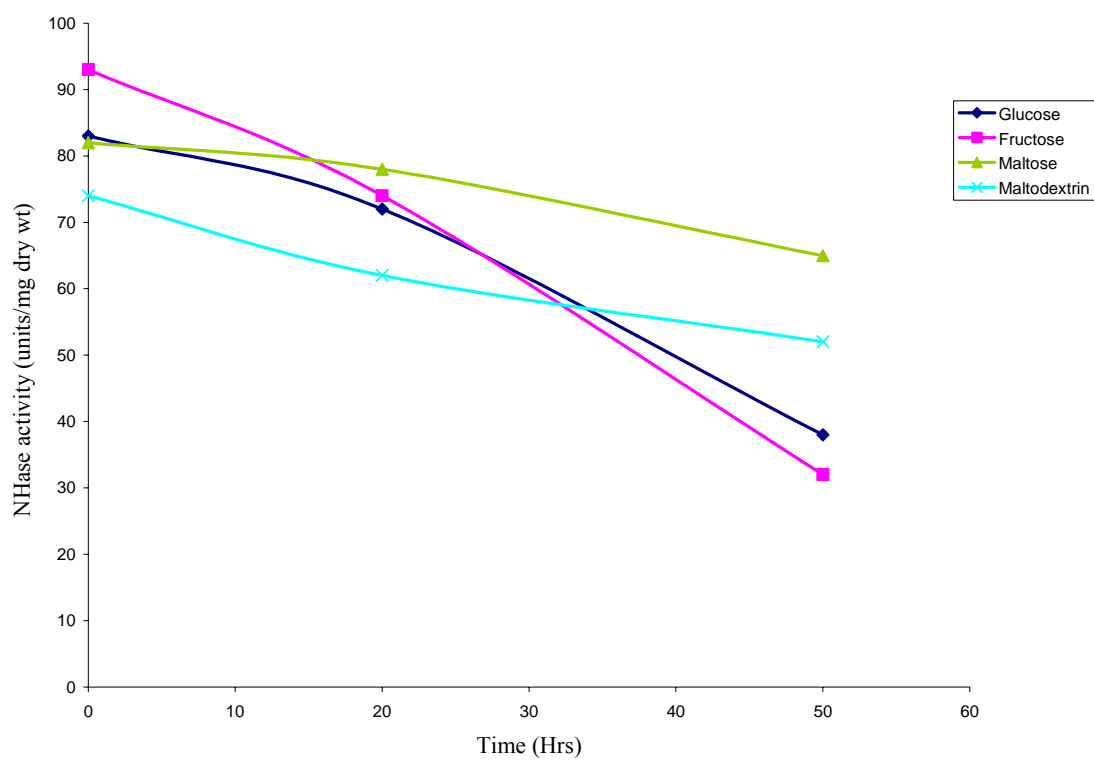


Fig 8. NHase stability at 55 °C of cells grown on media supplemented with trehalose. *Rhodococcus* sp DAP 96253 was grown on YEMEA supplemented with glucose (4g/L), fructose (4g/L), fructose (4g/L), maltodextrin (4g/L), cobalt and urea were added as inducers and trehalose (4g/L) as a stabilizer.

Analysis of mycolic acid profiles

Supplementation of basal medium (YEMEA) with sugars such as glucose, maltose, fructose and maltodextrin resulted not only in changes in the level and specificity of NHase but the stability as well. In order to evaluate if these observed differences in NHase activity and stability might be related to the rhodococcal cell envelope, mycolic acids content and the composition of the cell envelope were examined. Both as negative control for mycolic acid production (*Candida albicans* C30) (Fig. 9) and a positive control (*Mycobacterium intracellulare* 13950) (Fig. 10) were incorporated into the analysis. In addition both a low and a high molecular weight (LMW, HMW) internal standard were used. However, with the rhodococcal samples the LMW standard interfered with the mycolic acid analysis, therefore it was not used. Samples were analyzed in duplicate and results were reproducible.

Visual examination of the mycolic acids patterns, and examination of total mycolic acid content by peak areas showed that supplementation of different sugars and inducers affected the cell envelope mycolic acid composition (Fig. 11-18, Table 13a, Table 13b). The figures are labeled 1-5 to represent clusters. Cells grown on YEMEA supplemented with glucose only, had peak clusters 2, 3, 4, with prominent double peaks (Fig.11). Cells grown on YEMEA supplemented with fructose only, had a characteristically different pattern with clusters 2, 3, 4 no longer having the prominent double peaks but having an increase in certain peaks within the clusters (Fig. 12). Cells grown on YEMEA supplemented with maltose only, produced clusters 2, 3, 4 with a pattern similar to glucose grown cells however peak areas were significantly increased

(Fig. 13), there was a pattern of two dominant peaks within the clusters (2, 3, 4) as opposed to one prominent peak within the clusters when cells were grown on fructose supplemented media. YEMEA with maltodextrin only, showed a mycolic acid profile that was similar to the profile observed with cells grown on maltose supplemented media (Fig. 14). To better show differences in mycolic acid fingerprints Fig. 20 and 21 shows chromatogram overlays of Fig. 11-14. Lower peak areas were observed for all peak clusters when cells were grown on glucose and fructose as opposed to maltose and maltodextrin. Also increase and decrease of peaks within the various clusters can be seen in the chromatogram overlays.

The effect of inducers (cobalt and urea) on the mycolic acid patterns of *Rhodococcus* DAP 96253 was examined. The addition of cobalt and urea changed the mycolic acid pattern significantly in the glucose supplemented media (Fig. 15); clusters 2, 3 and 4 had a predominant single peak similar to the profile observed when cells were grown on un-induced media supplemented with fructose. Growth on induced media supplemented with fructose did not result in any significant changes to the mycolic acid profiles (Fig. 16). Clusters 2, 3 and 4 still had single predominant peaks, however peak areas had increased. Mycolic acid profiles produced when cells were grown on YEMEA supplemented with maltose or maltodextrin induced with cobalt and urea, changed significantly (Fig. 17 and 18); clusters 2, 3 and 4 had shown characteristic double peaks without cobalt and urea, however media induced with cobalt and urea produced profiles that had predominant single peaks within the clusters. Fig. 22 and 23 shows chromatogram overlays of the mycolic acid profiles seen in Fig. 15-18. These

overlays show that fructose grown cells had the greatest peak areas for clusters 2, 3, 4 while maltose had the least, these differences in peak areas represent differences in the amount of mycolic acid present in the cell envelope. The pattern observed with induced cells was different from the pattern observed in un-induced cells. Mycolic acid profile of un-induced cells showed that fructose or glucose supplementation led to decreased peak areas, while maltose or maltodextrin supplementation led to increased peak areas.

Growth in the presence of cobalt and urea influenced mycolic acid profiles in *Rhodococcus* sp DAP 96253, however it was unclear whether it was the cobalt, urea or both that were eliciting the changes in mycolic acid profiles. This was investigated by growing *Rhodococcus* sp DAP 96253 on YEMEA supplemented with glucose only, YEMEA supplemented with glucose and cobalt and YEMEA supplemented with glucose, cobalt and urea (Fig. 19) and examining the mycolic acid profiles. Cobalt had induced changes in mycolic acid profiles as there were changes in clusters 2, 3, 4 when compared to the profile observed when YEMEA was only supplemented with glucose. Peaks within clusters 2, 3, 4, increased significantly, the same pattern observed when cells were grown on YEMEA supplemented with glucose, cobalt and urea. This showed that the addition of cobalt without urea could lead to changes in mycolic acid profiles. The results indicate that carbohydrates and metals can affect the rhodococcal cell envelope, particularly the mycolic acids.

Percentage change in mycolic acid content was examined by comparing the summation of peak areas between RT 23 and 30 mins (Table 13a, 13b). Cells grown on YEMEA without cobalt and urea, supplemented with fructose, maltose, or maltodextrin

resulted in reduced mycolic acid content in the cell envelope. The addition of cobalt and urea to YEMEA supplemented with glucose or fructose resulted in a significant increase in mycolic acid content.

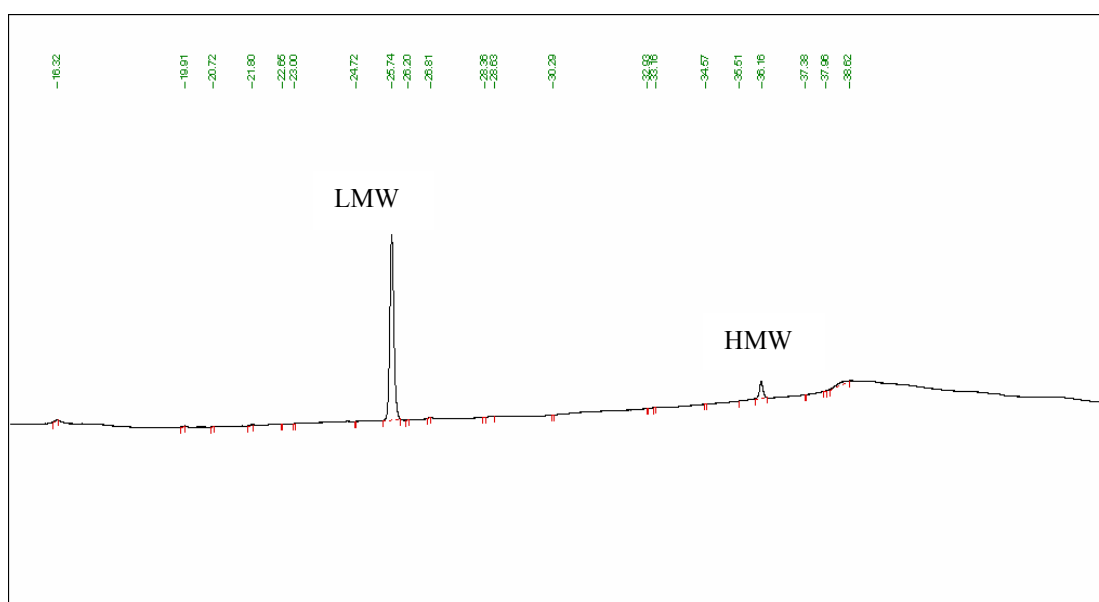


Fig. 9. Chromatogram of mycolic acid extract from *Candida albicans* C30 (negative control) grown on Lowenstein Jenson slants, showing LMW and HMW standards, no mycolic acids are observed between LMW and HMW.

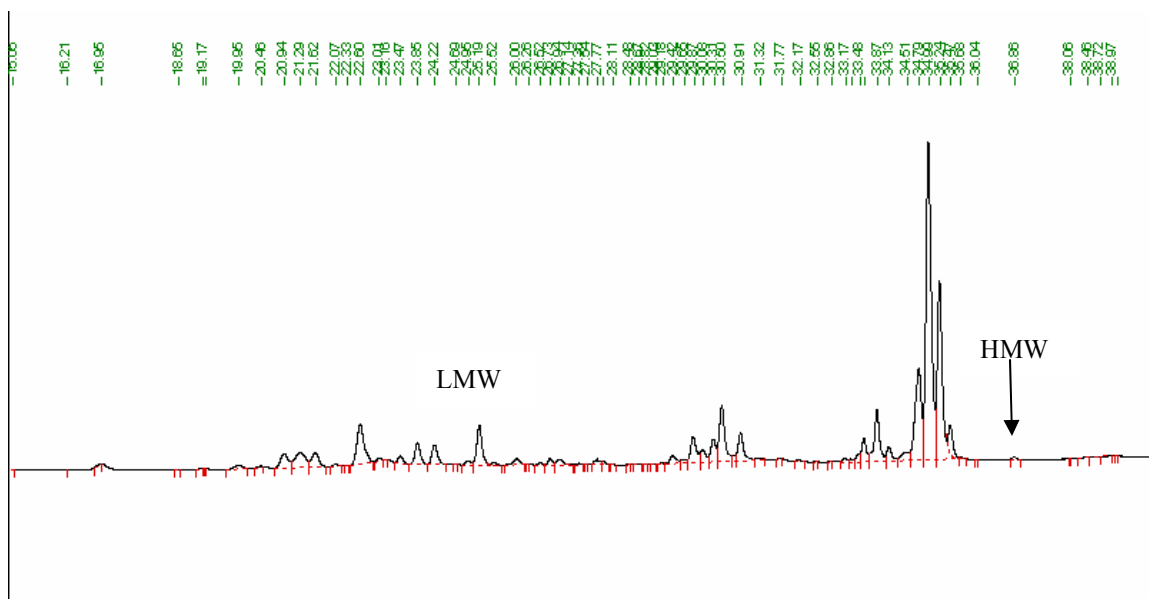


Fig. 10. Chromatogram of mycolic acid extract from *Mycobacterium intracellulare* 13950 (positive control) grown on Lowenstein Jenson slants, showing mycolic acids peaks and high molecular weight standard (HMW) .

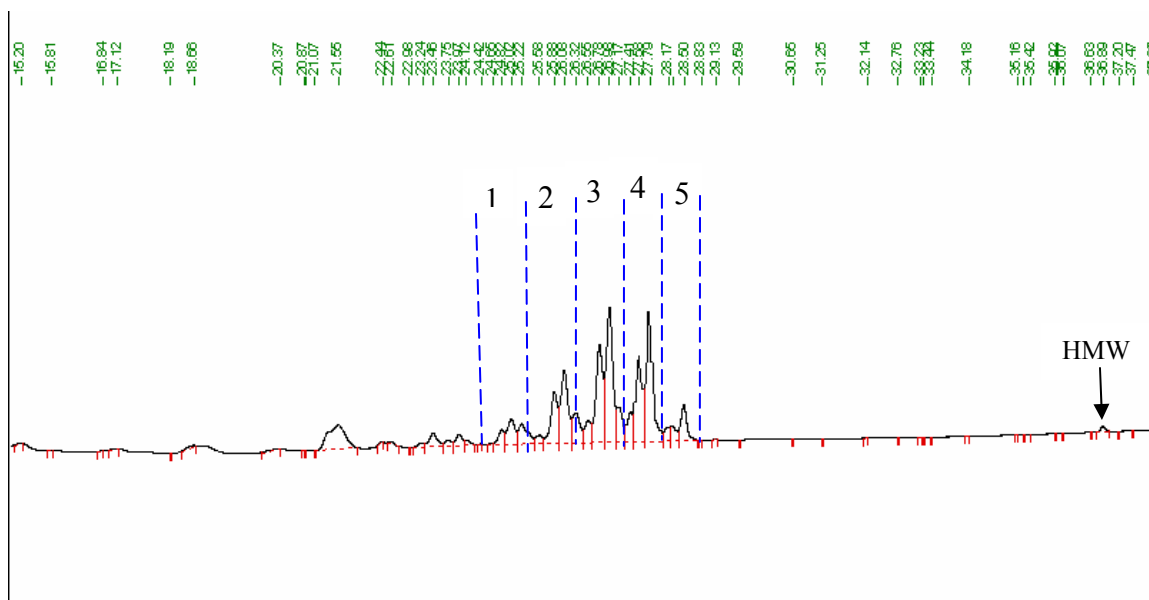


Fig. 11. Chromatogram of mycolic acid extract from *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with glucose (4g/L) showing peaks clusters 1-5 and high molecular weight standard (HMW).

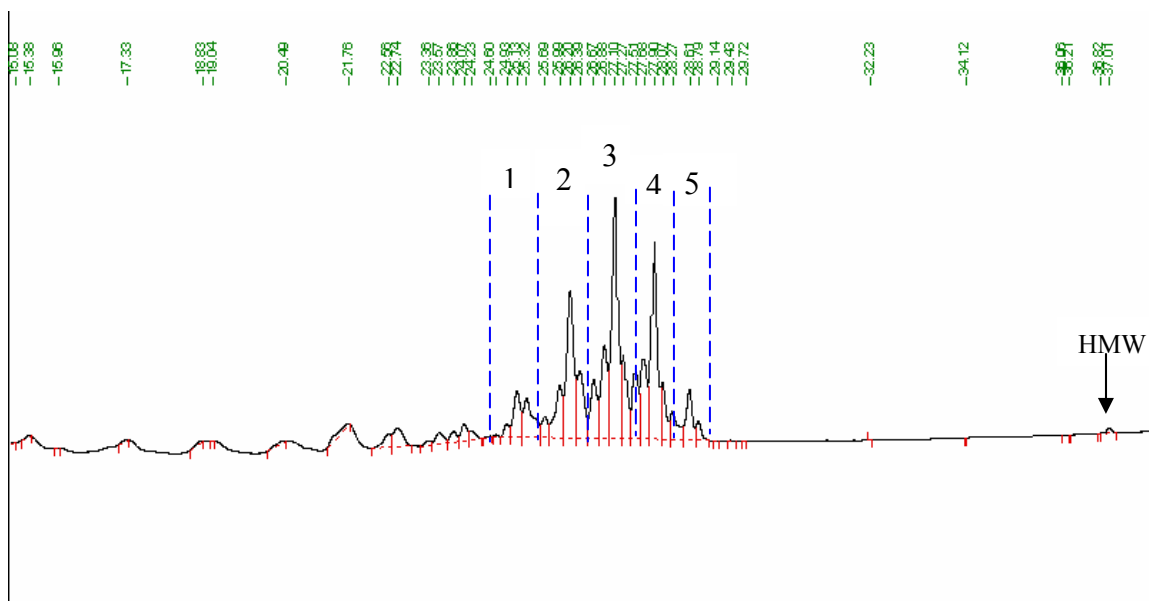


Fig. 12. Chromatogram of mycolic acid extract from *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with fructose (4g/L) showing peak clusters 1-5 and high molecular weight standard (HMW).

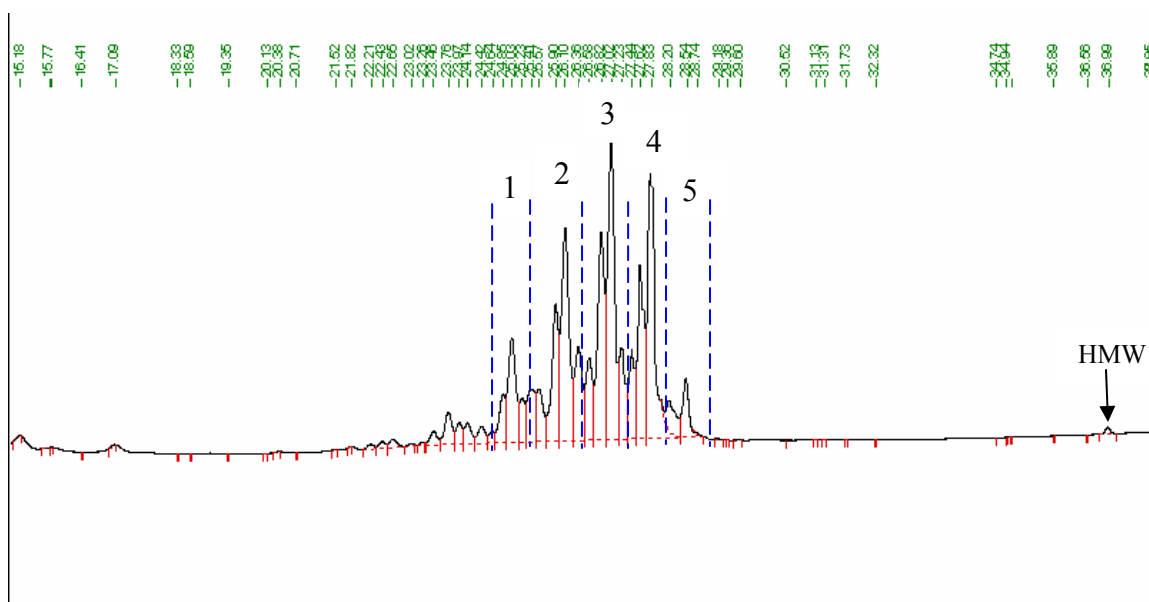


Fig. 13. Chromatogram of mycolic acid extract from *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with maltose (4g/L) showing peak clusters 1-5 and high molecular weight standard (HMW).

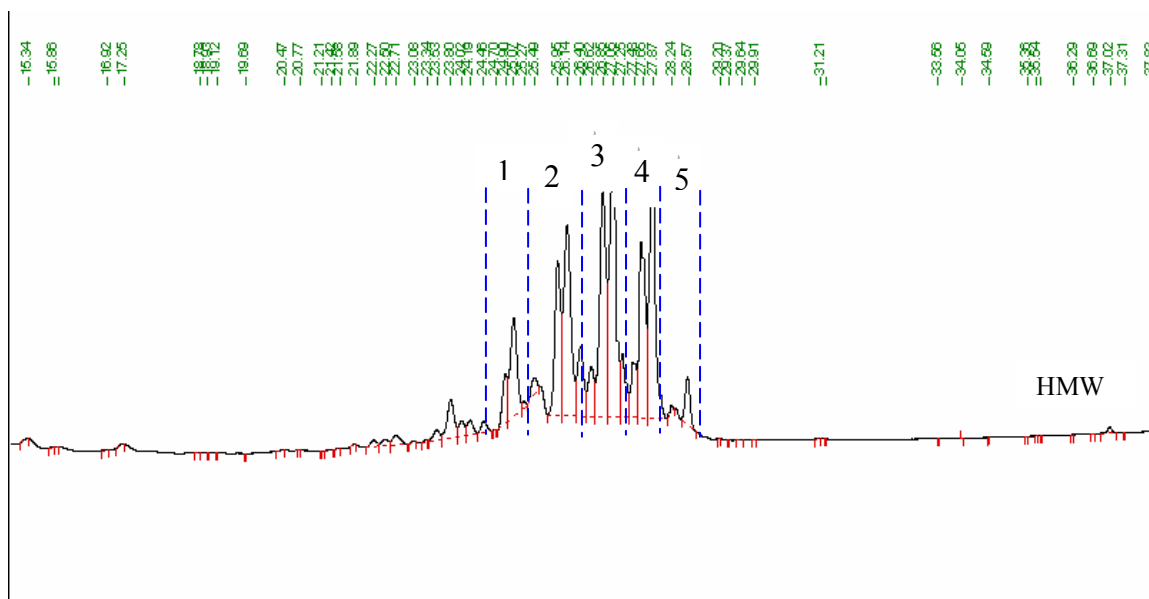


Fig. 14. Chromatogram of mycolic acid extract from *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with maltodextrin (4g/L) (dextrose equivalent =25) (4g/L) showing peak clusters 1-5 and high molecular weight standard (HMW).

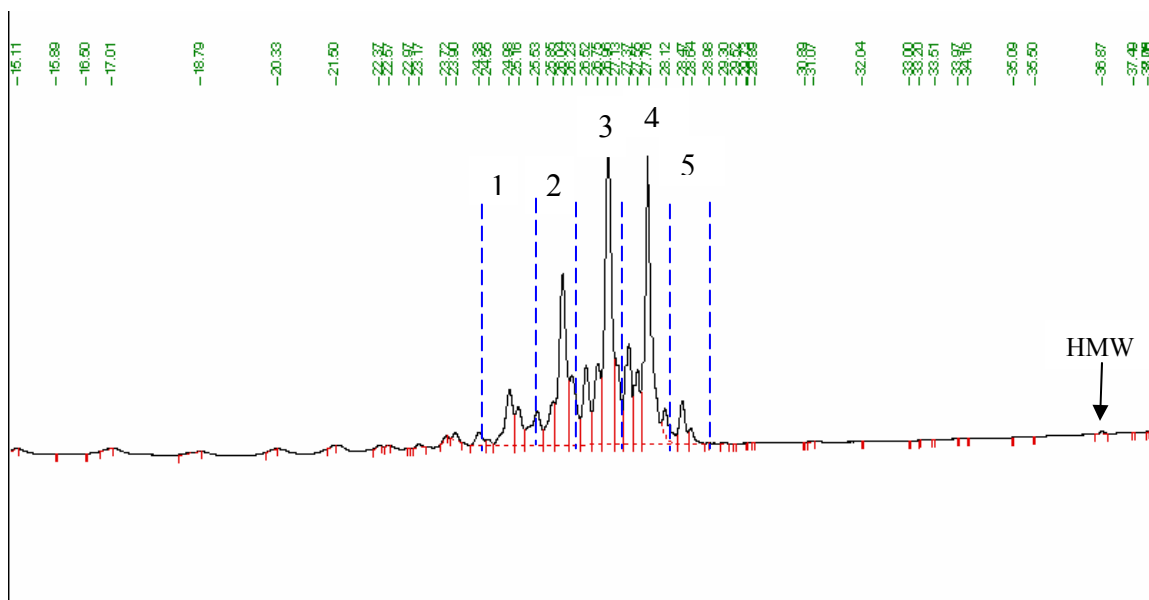


Fig. 15. Chromatogram of mycolic acid extract from *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L) showing peak clusters 1-5 and high molecular weight standard (HMW).

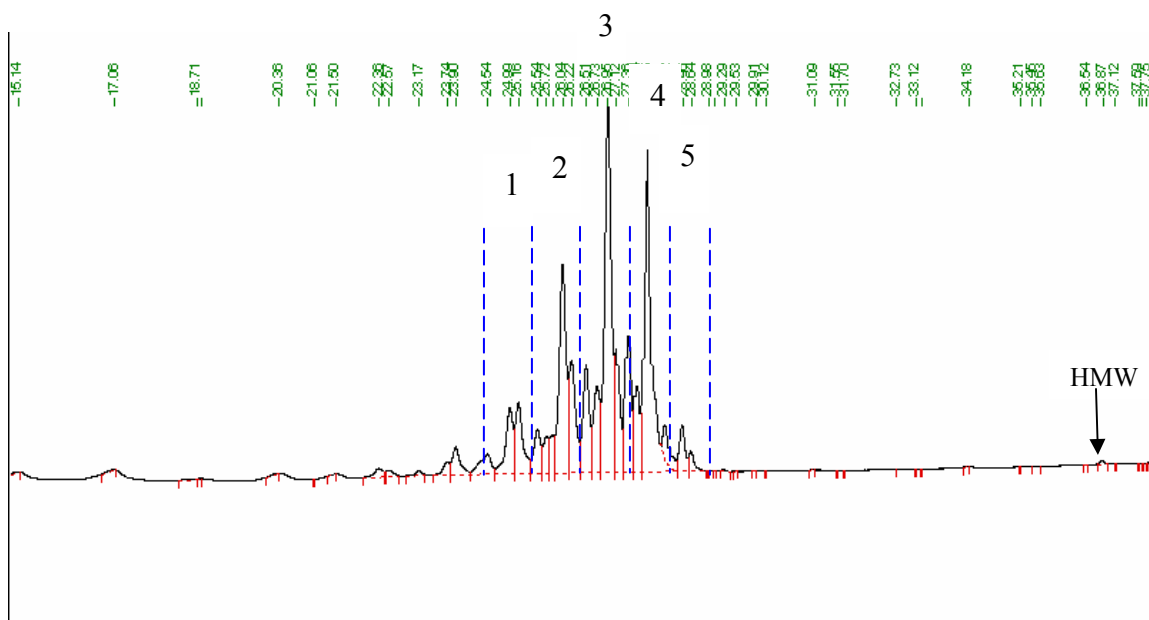


Fig. 16. Chromatogram of mycolic acid extract from *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with fructose (4g/L), cobalt (50mg/L) and urea (7.5g/L) showing peak clusters 1-5 and high molecular weight standard (HMW).

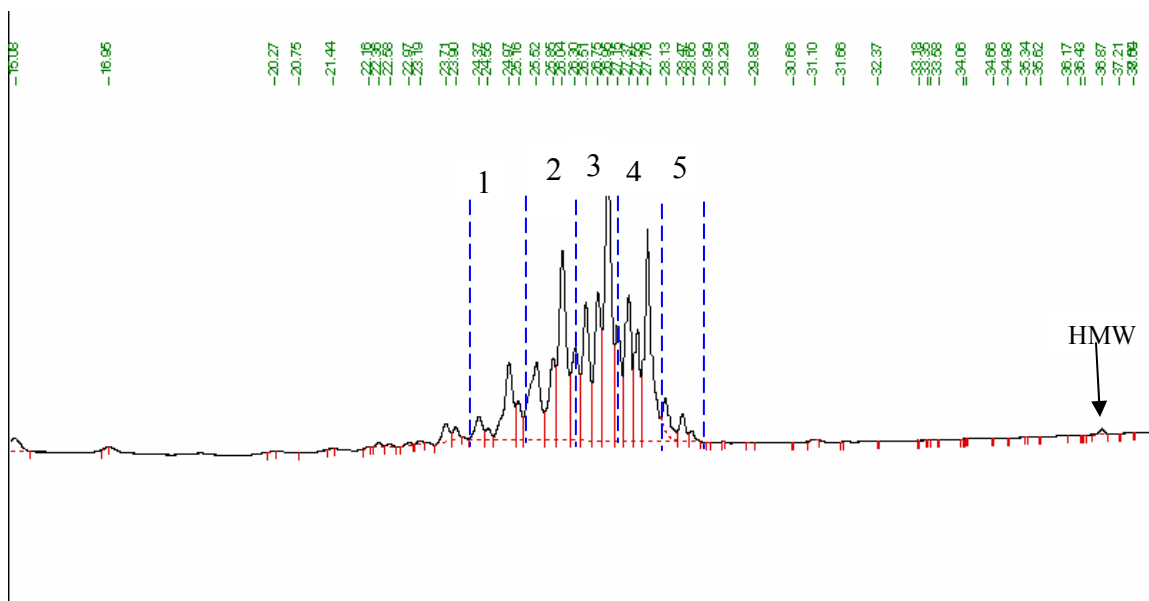


Fig. 17. Chromatogram of mycolic acid extract from *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with maltose (4g/L), cobalt (50mg/L) and urea (7.5g/L) showing peak clusters 1-5 and high molecular weight standard (HMW).

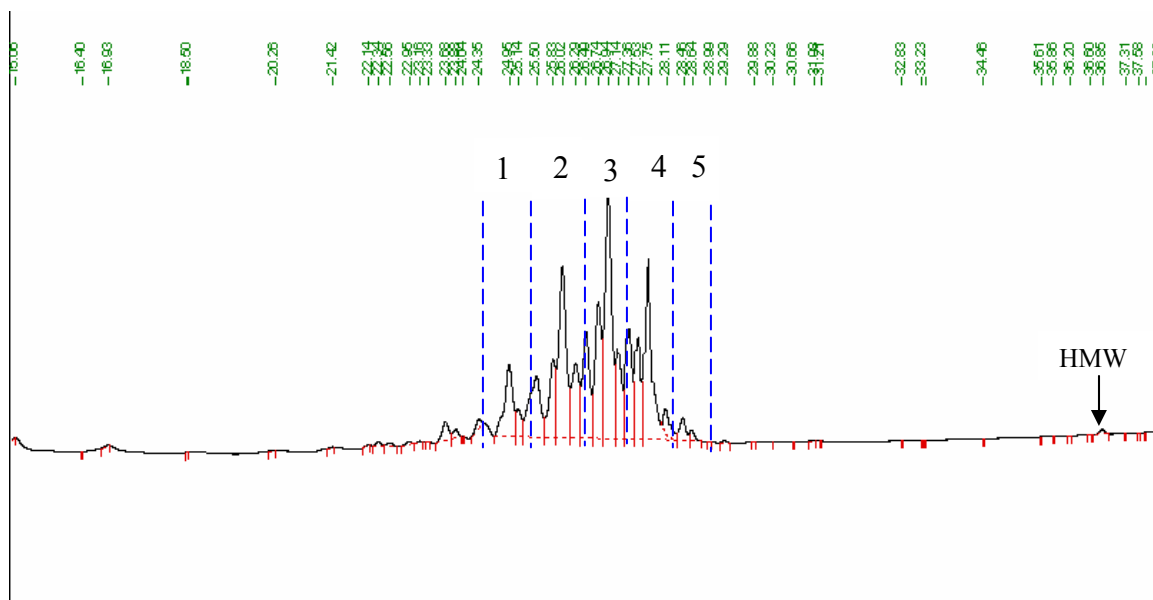
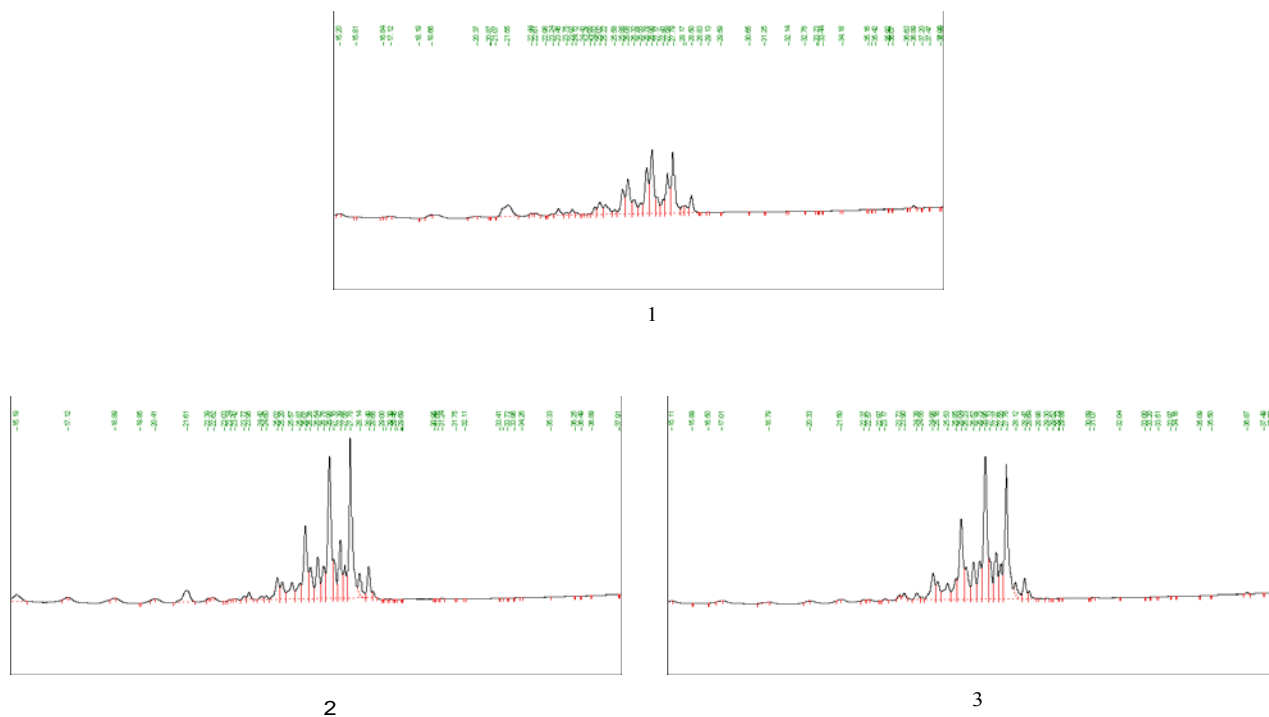


Fig. 18. Chromatogram of mycolic acid extract from *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with maltodextrin (4g/L), cobalt (50mg/L) and urea (7.5g/L) showing peak clusters 1-5 and high molecular weight standard (HMW).



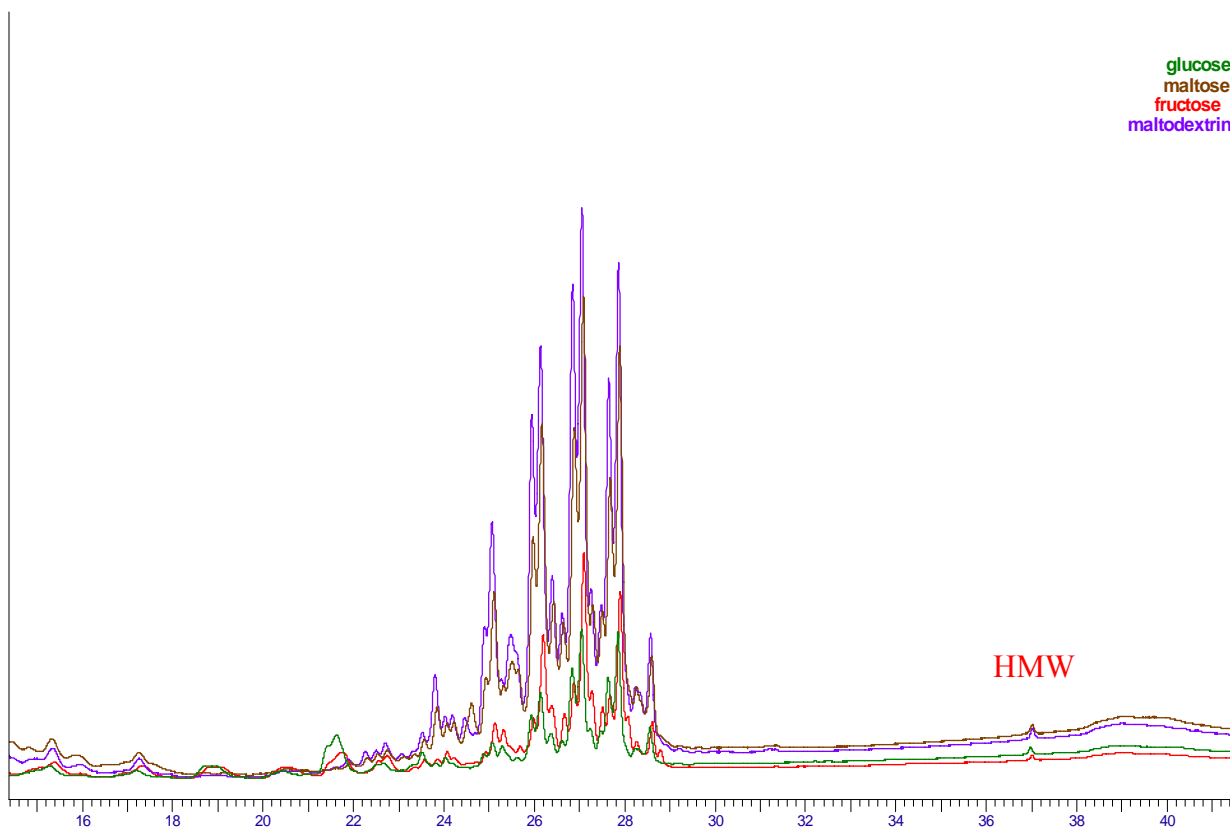


Fig. 20. Overlays of mycolic acid profiles of *Rhodococcus* sp DAP 96253 grown on YEMEA with supplementation of different sugars [glucose, maltose, fructose and maltodextrin (4g/L)].

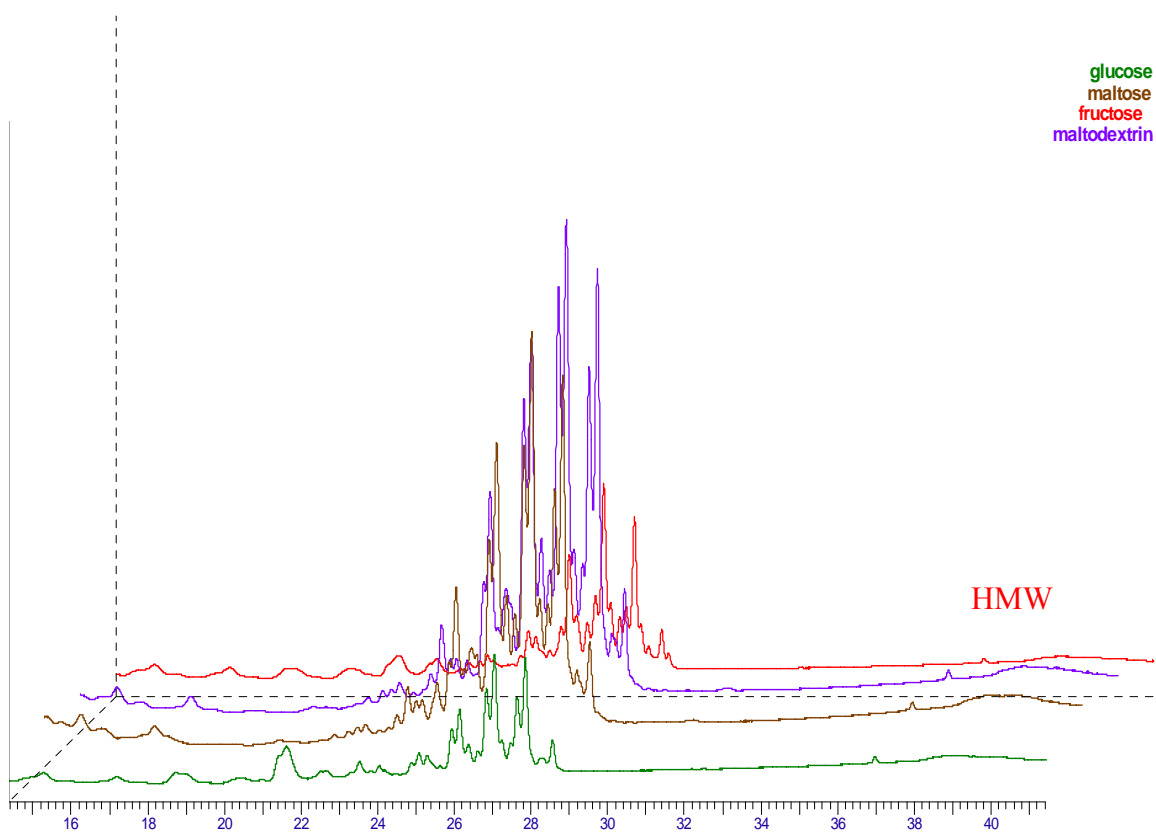


Fig. 21. 3-D view of chromatogram overlays seen in Fig. 17 of mycolic acids profiles of *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with different sugars [glucose, fructose, maltose and maltodextrin (4g/L)]
HMW – high molecular weight standards

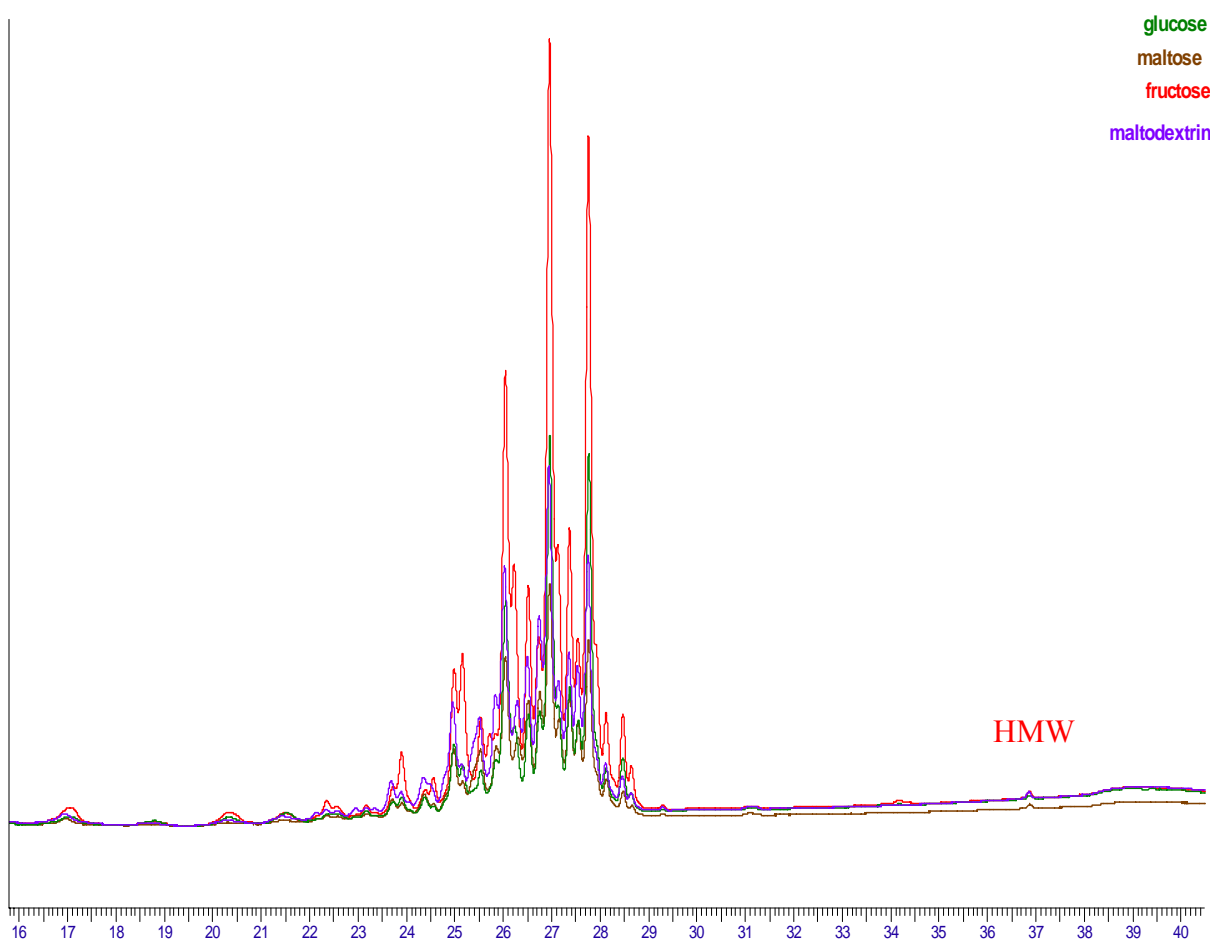


Fig 22. Overlays of mycolic acid profiles of *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with different sugars [glucose, fructose, maltose, maltodextrin (4g/L)] and inducers, cobalt (50mg/L) and urea (7.5g/L). HMW- high molecular weight standard.

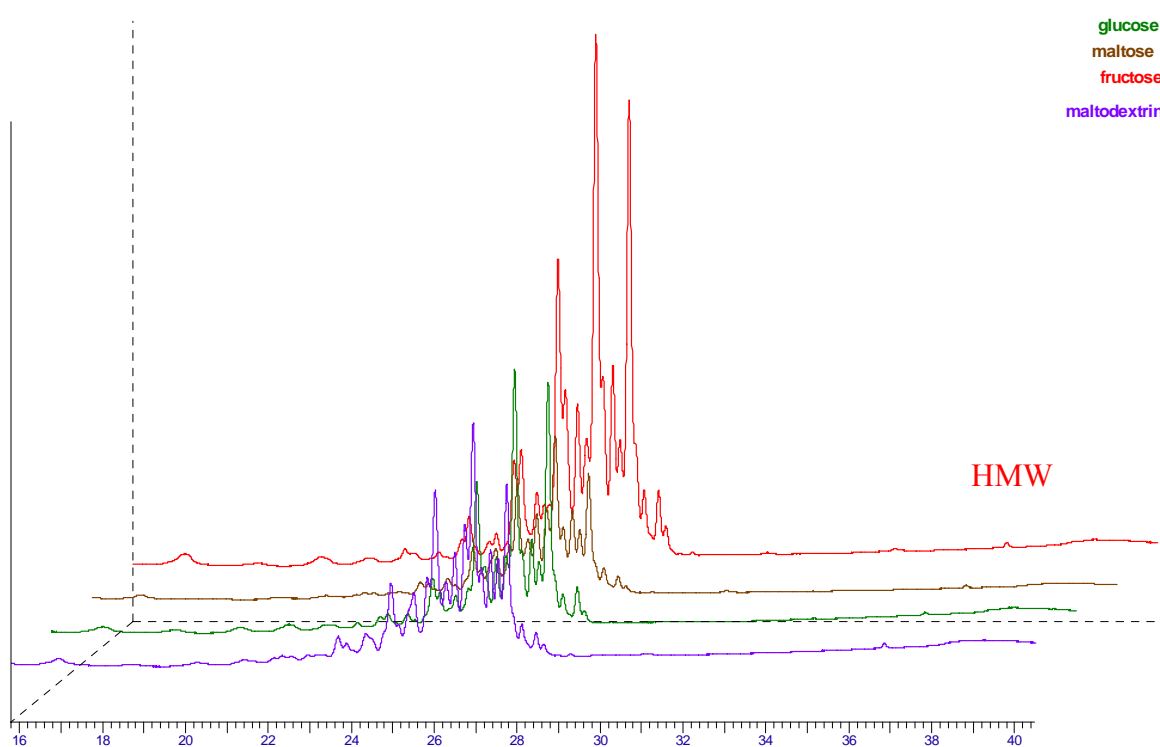


Fig. 23. 3-D view of chromatograms seen in Fig 19- overlays of mycolic profiles of *Rhodococcus* DAP 96253 grown on YEMEA supplemented with different sugars [glucose, fructose, maltose, maltodextrin (4g/L)] and inducers, cobalt (50mg/L) and urea (7.5g/L), HMW- high molecular weight standard.

Table 13a. Percentage change in total mycolic acid^a due to supplementation of different sugars in YEMEA non induced and induced for Nitrile Hydratase

| Media | % change in mycolic acid content |
|------------------------|----------------------------------|
| F ^b | 14% decrease |
| M ^b | 210% increase |
| MD ^b | 235% increase |
| F, Co, U ^c | 3% decrease |
| M, Co, U ^c | 19% decrease |
| MD, Co, U ^c | 34% decrease |

F- YEMEA supplemented with fructose (4g/L)

M- YEMEA supplemented with maltose (4g/L)

MD- YEMEA supplemented with maltodextrin (4g/L)

G, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

F, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

M, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

MD, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

a- calculated by summing peak areas between retention times 23 and 30mins

b- compared to mycolic acid content of cells grown on YEMEA supplemented with glucose(4g/L)

c.- compared to mycolic acid content of cells grown on YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

Table 13b. Percentage change in total mycolic acid^a due to supplementation of cobalt and urea to YEMEA with different sugars for induction of Nitrile Hydratase

| Media | % change in mycolic acid content |
|-----------|----------------------------------|
| G, Co, U | 240% increase |
| F, Co, U | 280% increase |
| M, Co, U | 12% decrease |
| MD, Co, U | 34% decrease |

G, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

F, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

M, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

MD, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

a- calculated by summing peak areas between retention times 23 and 30min

Analysis of glycolipids

While the addition of different sugars and/or inducers resulted in changes in cell envelope mycolic acids. It was further hypothesized that there also could be changes in the glycolipid content of the cell envelope. Total lipids were extracted from *Rhodococcus* sp DAP 96253 and separated on a silica gel plates. Glycolipids were visualized by spraying with 1-naphthol followed by heating (Fig. 24). While none of the visualized spots from the extracts corresponded to the position the standard [trehalose dimycolate (TDM) purified from *Mycobacterium tuberculosis*] it could be inferred that they were different glycolipids present as the standard also turned purple upon spraying with 1- naphthol. Cells that were un-induced 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.

Cells that were induced for NHase all had glycolipid spots that were close to the origin, a second spot was significantly increased in cells grown on glucose, fructose, or maltose. Cells induced and grown on maltodextrin also showed this second glycolipid spot, but it did not show up in the image. Extracts from cells that were induced and grown in the presence of fructose, had a third glycolipid spot that was not seen with any of the other extracts. Cells grown on YEMEA supplemented with fructose and induced with cobalt and urea showed the greatest diversity of glycolipids. The supplementation of different sugars and addition of inducers such as cobalt and urea to YEMEA also affected the type of glycolipids produced and the quantity present in the cell envelope of *Rhodococcus* sp DAP 96253.

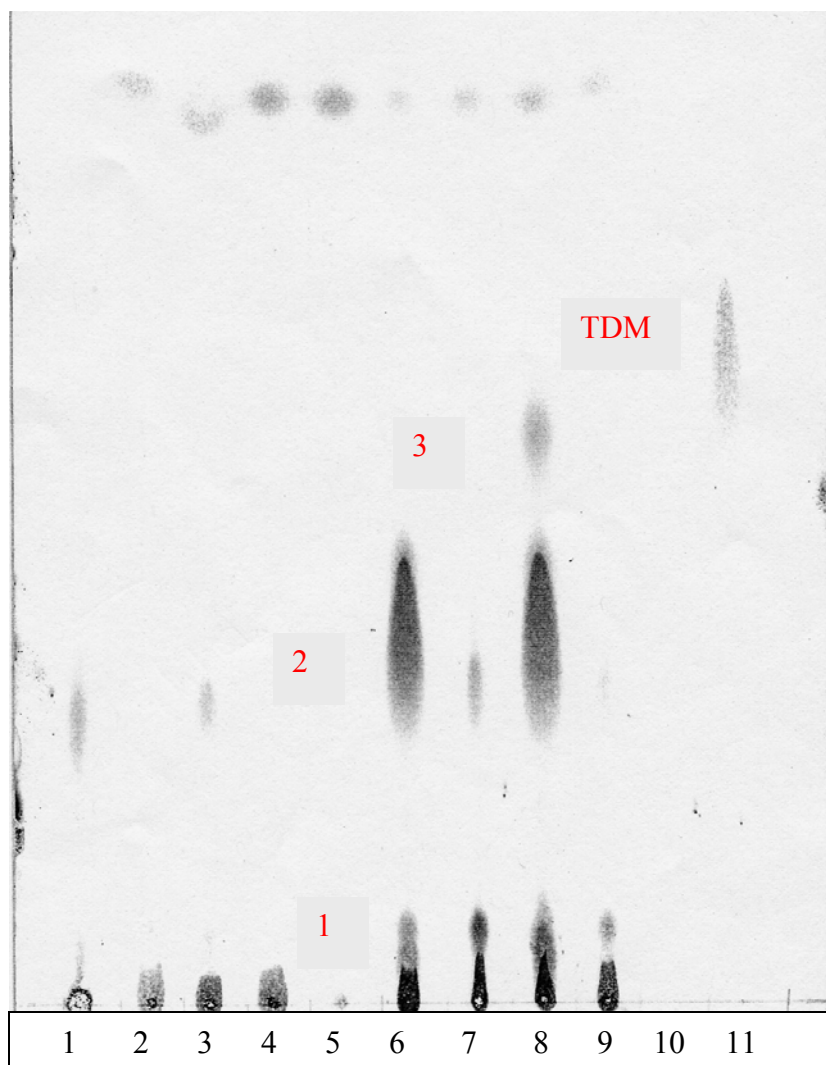


Fig. 24. TLC of total lipid extracts sprayed with 1-naphthol to visualize glycolipids. *Rhodococcus* sp DAP 96253 was grown on YEMEA supplemented with, (lane 1) glucose (4g/L) only, (lane 2) maltose (4g/L) only, (lane 3) fructose (4g/L) only, (lane 4) maltodextrin (4g/L) only (25 DE), (lane 6) glucose (4g/L) cobalt (50mg/L), and urea (7.5g/L), (lane 7) maltose (4g/L) cobalt (50mg/L) and urea (7.5g/L), (lane 8) fructose (4g/L) cobalt (50mg/L) and urea (7.5g/L), (lane 9) maltodextrin (4g/L) cobalt (50mg/L) and urea (7.5g/L), lane 10 was empty and lane 11 contained trehalose dimycolate (TDM) purified from *Mycobacterium tuberculosis*.

Analysis of surface proteins

S-layer proteins have been identified in *Corynebacterium*. Electron microscopy analysis of freeze fracture preparations was used to determine if such ordered arrangements of proteins existed on the surface of the *Rhodococcus* sp DAP 96253.

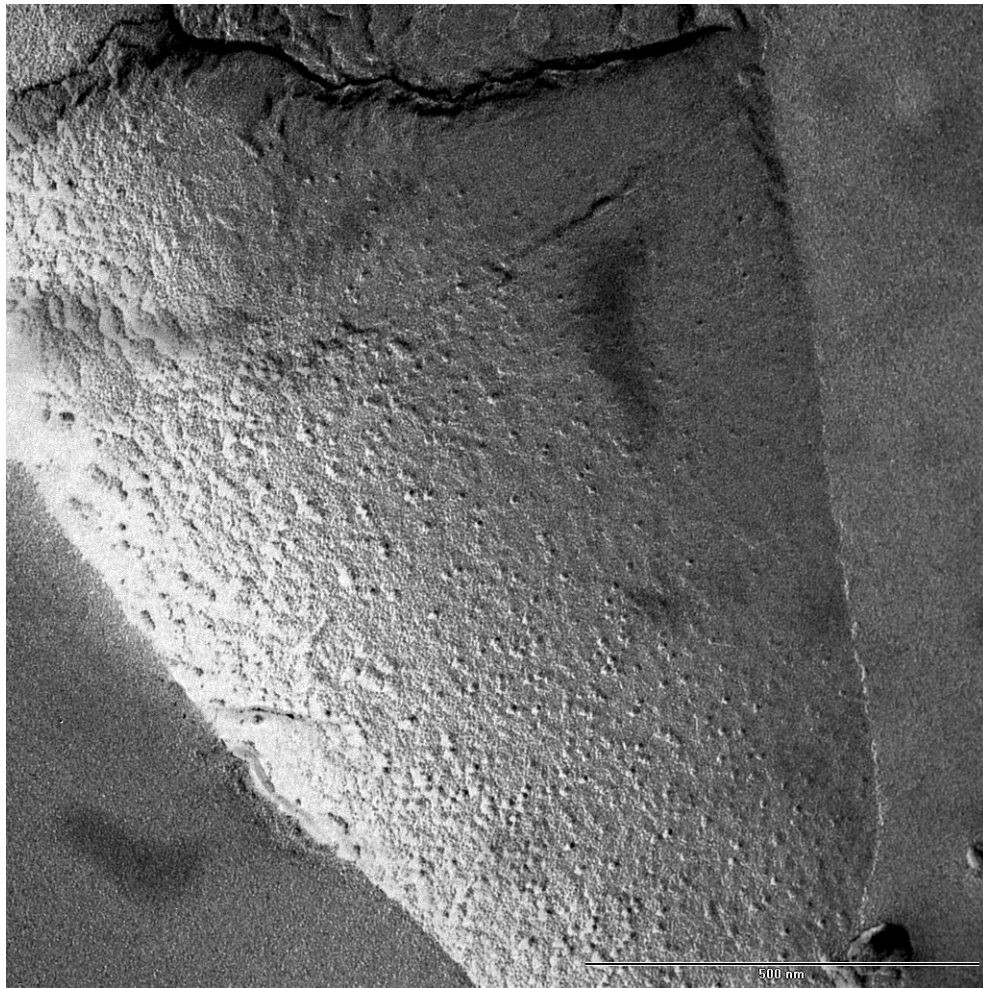


Fig 25. Electron micrograph of the freeze fracture preparation of *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L).

The surface of *Rhodococcus* sp DAP 96253 after growth on YEMEA supplemented with the different sugars(4g/L), cobalt (50mg/L) and urea (7.5g/L) did show many surface proteins however these surface proteins were not present in any particular structured array typical of S-layers (Fig 25 and 26).

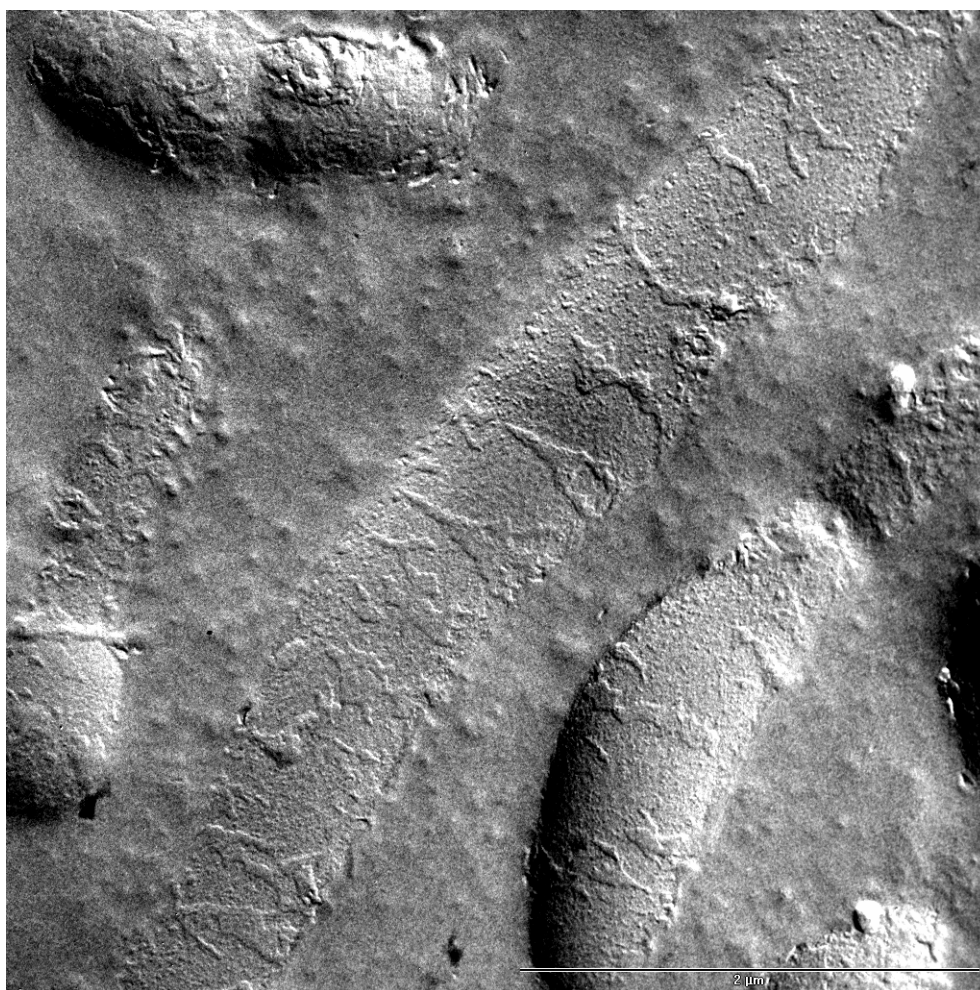


Fig. 26. Electron micrograph of freeze fracture preparation of *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with maltose (4g/L), cobalt (50mg/L), and urea (7.5g/L).

Rhodococcus sp DAP 96253 grown on YEMEA supplemented with maltose (4g/L), cobalt (50mg/L) and urea (7.5g/L) did not show the presence of a S- Layer, but the surface did have an arrangement of clumps that were not observed on the surface of the cells grown on YEMEA supplemented with the other sugars(Fig. 26).

A method for S-layer extraction for *Corynebacterium glutamicum* was used with *Rhodococcus* sp DAP 96253 grown on YEMEA with and without inducers for NHase and supplemented with glucose, fructose, maltose or maltodextrin. The extracted proteins were analyzed by SDS-PAGE (Fig. 27) and some were identified by MALDI TOF/TOF. Cellular proteins and cell envelope proteins were identified. NHase subunits α and β were identified as well as a lipopolysaccharide biosynthesis glycosyl transferase, and a 4- COG 1629 outer membrane receptor protein involved in metal transport.

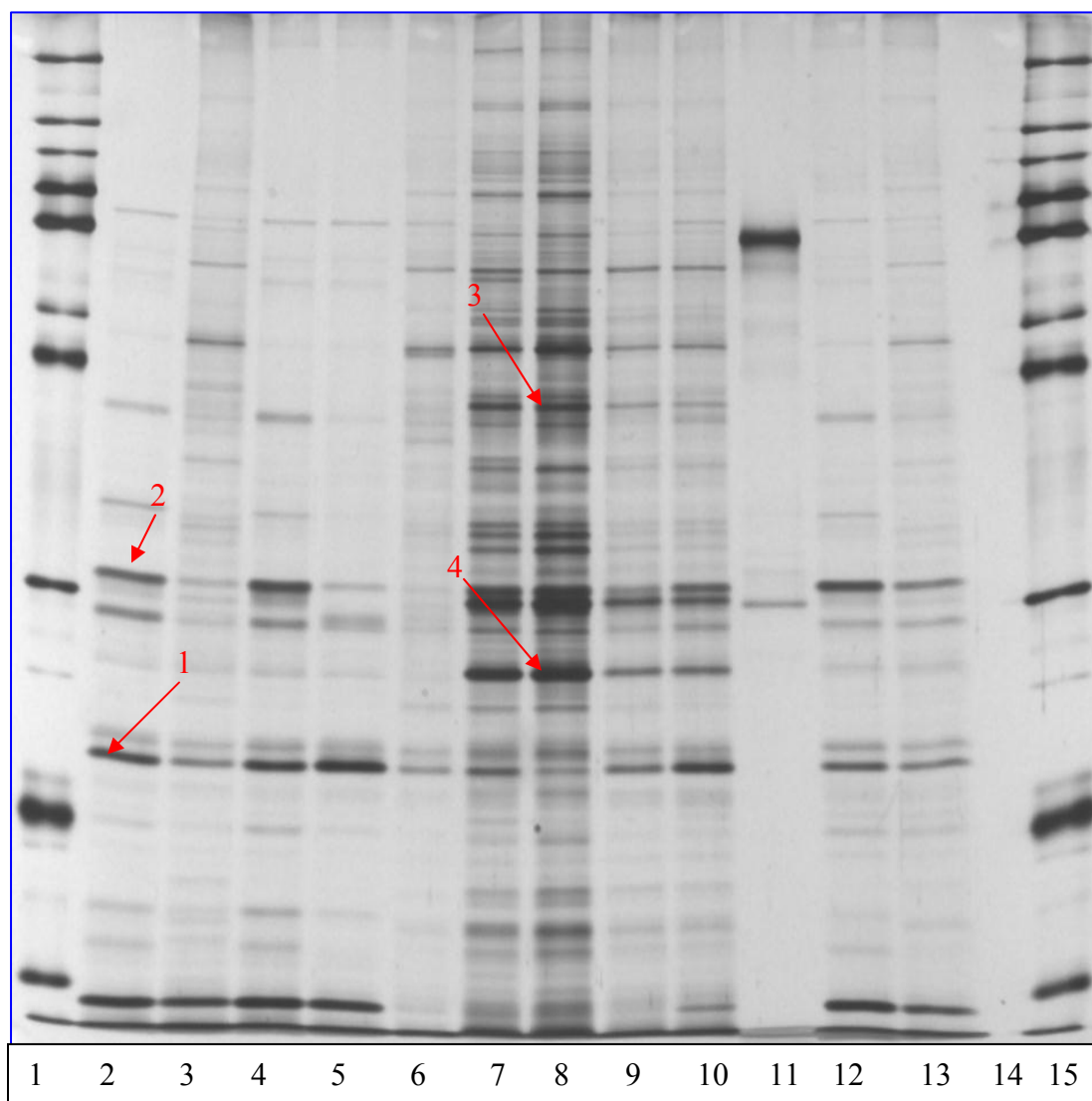


Fig 27. Gel electrophoresis of cell envelope extracts of *Rhodococcus* sp DAP 96253 grown on lane 2- YEMEA supplemented with glucose (4g/L), lane 3- with maltose (4g/L), lane 4- with fructose (4g/L), lane 5- maltodextrin (4g/L), lane 7- glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L), lane 8- fructose (4g/L), cobalt (50mg/L) and urea (7.5g/L), lane 9- maltose(4g/L), cobalt (50mg/L) and urea(7.5g/L), lane 10- maltodextrin (4g/L) , cobalt (50mg/L) and urea (7.5g/L), lane 11- *Mycobacterium* extract. Lane 1 and 15- molecular weight markers. Arrows 1- 4 indicate protein bands that were cut out subjected to trypsin digest and identified by MALDI-TOF/TOF , 1- α NHase, 2- β NHase, 3- Lipopolysaccharide biosynthesis glycosyl transferase, 4- COG 1629 outer membrane receptor protein-metal transport

Analysis of trehalose

Initially trehalose within the cell and the cell envelope was measured by the anthrone reaction. Cellular trehalose levels were higher in un-induced cells than induced cells for all conditions except for fructose supplementation (Table 14), e.g. cells grown with supplemented glucose had a cellular trehalose concentration of 6000 mg/g cdw, cells grown on media supplemented with glucose, cobalt and urea had 4500 mg/g cdw of cellular trehalose. Cellular trehalose concentration was highest when YEMEA was supplemented with glucose or maltose [un-induced- 6000 mg/g cdw (glucose) and 6500 mg/g cdw (maltose), induced- 4500 mg/g cdw (glucose) and 5550 mg/g cdw (maltose)]. Fructose supplemented media produced the lowest cellular trehalose concentration in un-induced cells and induced cells (4200 mg/g cdw and 2200 mg/g cdw respectively). Cellular trehalose levels were higher than lipid bound trehalose levels except for induced YEMEA supplemented with fructose which had a trehalose concentration of 2200 mg/g cdw inside the cell and 4400 mg/g cdw in the cell envelope.

Gas Chromatography was used as an alternative method to the anthrone reaction for specific trehalose analysis. For GC analysis of trehalose more cells were used and larger extraction volumes were required. Cells grown on YEMEA induced with cobalt and urea and supplemented with different sugars (glucose, fructose, maltose, maltodextrin) were analyzed for their cellular trehalose and lipid bound trehalose content. Previous work had shown that asparagine (ASN) was an inducer of NHase. The effect of this amino acid on cellular trehalose and lipid bound trehalose was

investigated. Trehalose is known as stabilizer of proteins (Sola-Penna et al., 1997) hence the effect of additional supplementation of trehalose on cellular trehalose and lipid bound trehalose was also assessed. Fig. 28 and 29 show the chromatograms of derivatized cellular and cell envelope extracts from *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with glucose, cobalt and urea, trehalose had a retention time of 15.9 min. shown by the arrows on the figures.

GC analysis showed that cellular trehalose did change significantly with the supplementation of different sugars in YEMEA with cobalt and urea. Table 15 shows that supplementation of fructose and maltose made a statistical different in cellular trehalose levels compared to cells grown on YEMEA supplemented with glucose. When ASN was added to growth media trehalose levels increased significantly from 595 mg/g cdw to 1523 mg/g cdw in maltose supplemented YEMEA and 2083 mg/g cdw in maltodextrin supplemented YEMEA. Supplementation of trehalose in the media increased cellular trehalose levels for glucose, fructose, maltose and maltodextrin grown cells.

Cells grown on media supplemented with fructose, maltose or maltodextrin, cobalt and urea had significantly higher levels of trehalose on the outside of the cell than on the inside, particularly the cells grown on media supplemented with fructose (280 mg/g cdw cellular trehalose level compared to 8050 mg/g cdw present in the cell envelope, Table 15). Cells grown on YEMEA supplemented with glucose or fructose with cobalt, urea and ASN also had slightly higher trehalose levels in the cell envelope

than inside the cells. The addition of trehalose to YEMEA supplemented with the different sugars did not lead to increased levels of trehalose in the cell envelope.

Table 15 also shows p values and standard deviations for trehalose cellular concentrations and lipid bound trehalose. These values were derived by comparing all trehalose concentrations with the trehalose concentration of cells grown on YEMEA supplemented with glucose, cobalt and urea. P-values greater than 0.05 were statistically insignificant.

Cellular and lipid bound levels of trehalose measured by the anthrone reaction were higher than trehalose levels measured by GC analysis, however similar trends were observed i.e. cells grown on fructose with or without inducers had the lowest levels of cellular trehalose, lipid bound trehalose was increased when cells were grown on media supplemented with fructose, cobalt and urea.

Table 14– Cellular and lipid bound trehalose (mg/g cdw) in *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with different sugars and inducers measured by the anthrone reaction

| Supplement | Cellular Trehalose (mg/g cdw) | Lipid Bound Trehalose (mg/g cdw) |
|----------------------------|-------------------------------|----------------------------------|
| Glucose | 6000 | ND |
| Fructose | 4200 | ND |
| Maltose | 6500 | ND |
| Maltodextrin | 4600 | ND |
| Glucose, cobalt, urea | 4500 | 3500 |
| Fructose, cobalt, urea | 2200 | 4400 |
| Maltose, cobalt, urea | 5600 | 3500 |
| Maltodextrin, cobalt, urea | 4100 | 1600 |

ND- not determined

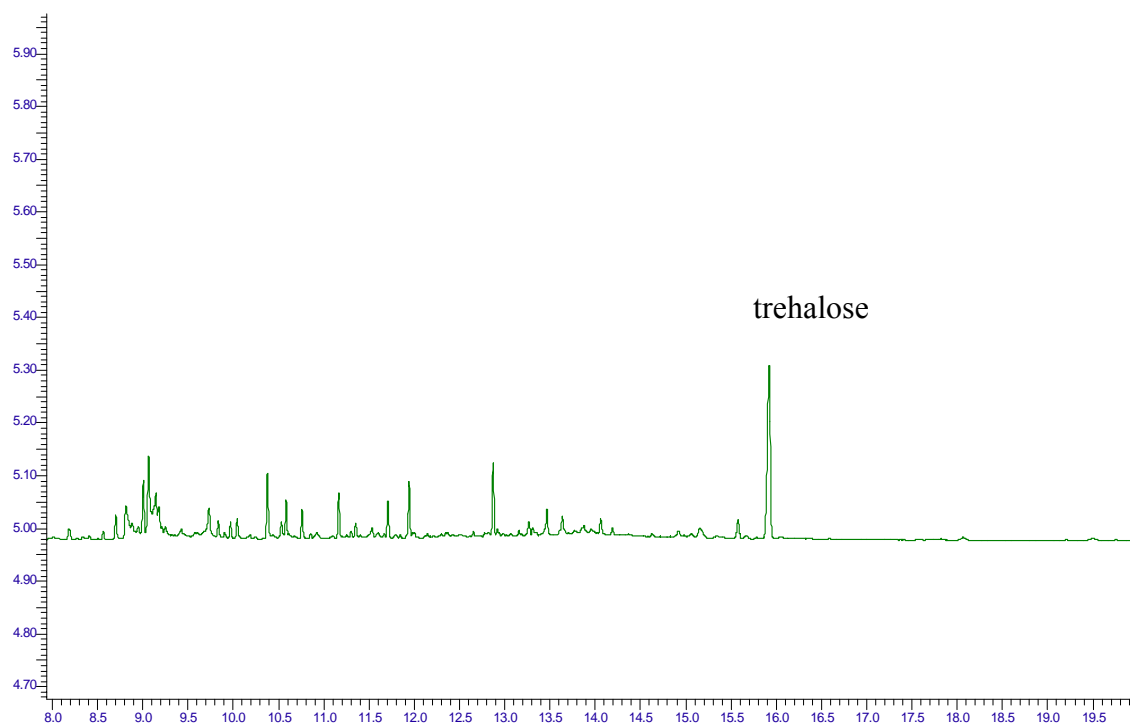


Fig 28. GC chromatogram of TCA extract from *Rhodococcus* DAP 96253 grown on YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

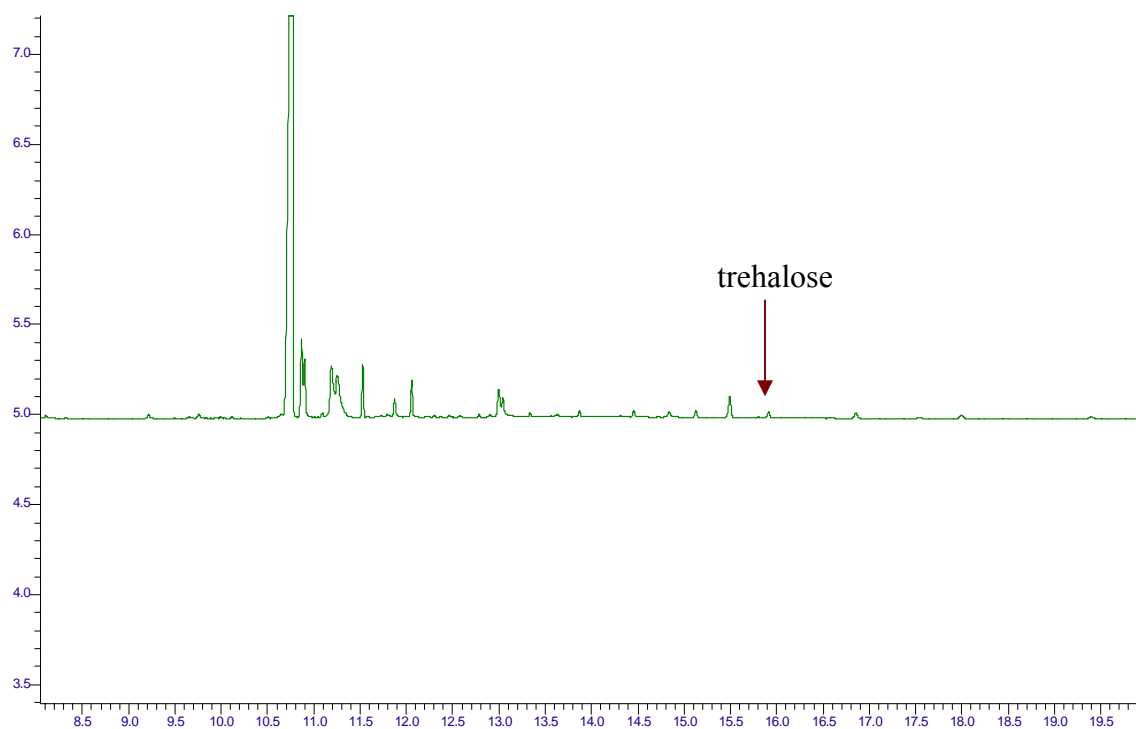


Fig 29. GC chromatogram showing trehalose released from cell envelope lipids of *Rhodococcus* DAP 96253 grown on YEMEA supplemented with glucose (4g/L) cobalt (50mg/L) and urea (7.5g/L)

Table 15. Statistical evaluation of cellular and lipid bound trehalose measured by GC in *Rhodococcus* sp DAP 96253 grown on YEMEA supplemented with different sugars and inducers

| Supplement | Cellular trehalose (mg/g cdw) | P Value | Stan. Dev. | Lipid bound trehalose (mg/g cdw) | P Value | Stan. Dev. | Initial NHase activity (units/mg cdw) |
|----------------|-------------------------------|---------------|------------|----------------------------------|--------------|------------|---------------------------------------|
| G, Co, U | 490 | 0 | 0 | 893 | 0 | 0 | 101 |
| F, Co, U | 280 | 0.003 | 134 | 8050 | 0.012 | 3537 | 120 |
| M, Co, U | 595 | 0.010 | 132 | 3220 | 0.030 | 1414 | 133 |
| MD, Co, U | 595 | 0.140 | 129 | 4900 | 0.012 | 2112 | 140 |
| G, Co, U, ASN | 543 | 0.210 | 91 | 910 | 0.710 | 125 | 100 |
| F, Co, U, ASN | 630 | 0.180 | 100 | 945 | 0.240 | 167 | 84 |
| M, Co, U, ASN | 1523 | 0.037 | 482 | 245 | 0.030 | 273 | 118 |
| MD, Co, U, ASN | 2083 | 0.004 | 806 | 1085 | 0.155 | 157 | 85 |
| G, Co, U, Tre | 963 | 0.034 | 343 | 429 | 0.002 | 242 | 96 |
| F, Co, U, Tre | 700 | 0.060 | 182 | 770 | 0.129 | 106 | 101 |
| M, Co, U, Tre | 8435 | 0.0003 | 4264 | 4165 | 0.014 | 1511 | 110 |
| MD, Co, U, Tre | 8400 | 0.001 | 4083 | 1025 | 0.060 | 78 | 112 |

continued on next page

G- YEMEA supplemented with glucose (4g/L)

F- YEMEA supplemented with fructose (4g/L)

M- YEMEA supplemented with maltose (4g/L)
 MD- YEMEA supplemented with maltodextrin (4g/L)
 G, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)
 F, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)
 M, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)
 MD, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)
 G, Co, U, ASN- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L), urea (7.5g/L) and asparagine (1g/L)
 F, Co, U, ASN- YEMEA supplemented with fructose (4g/L), cobalt (50mg/L), urea (7.5g/L) and asparagine (1g/L)
 M, Co, U, ASN- YEMEA supplemented with maltose (4g/L), cobalt (50mg/L), urea (7.5g/L) and asparagine (1g/L)
 MD, Co, U, ASN- YEMEA supplemented with maltodextrin (4g/L), cobalt (50mg/L), urea (7.5g/L) and asparagines (1g/L)
 G, Co, U, Tre- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L), urea (7.5g/L) and trehalose (4g/L)
 F, Co, U, Tre- YEMEA supplemented with fructose (4g/L), cobalt (50mg/L), urea (7.5g/L) and trehalose (4g/L)
 M, Co, U, Tre- YEMEA supplemented with maltose (4g/L), cobalt (50mg/L), urea (7.5g/L) and trehalose (4g/L)
 MD, Co, U, Tre- YEMEA supplemented with maltodextrin (4g/L), cobalt (50mg/L), urea (7.5g/L) and trehalose (4g/L)

Antibiotic susceptibility

The susceptibility of *Rhodococcus* sp DAP 96253 to various antibiotics (erythromycin, rifampin, tetracycline, ampicillin, vancomycin) after growth on un-inducing and inducing YEMEA supplemented with different sugars was examined as an indication of changes in the cell envelope. MICs ($\mu\text{g/ml}$) were determined for erythromycin and rifampin by reading the value from the scale on the Etest strip where the ellipse edge intersected the strip (Fig. 30 and 31, Table 16). Zone of inhibitions (mm) were measured for vancomycin (30 μg), ampicillin (10 μg), tetracycline (30 μg) (Fig. 33 and 34, Table 17).

MIC for erythromycin and rifampin were higher when *Rhodococcus* sp DAP 96253 was grown on un-inducing YEMEA supplemented with the different sugars as opposed to growth on inducing YEMEA supplemented with the different sugars. Cells that were grown on YEMEA containing cobalt and urea were more sensitive to the

antibiotics. There were also differences in the MIC when different sugars were used as supplements, for example cells grown on un-inducing YEMEA supplemented with fructose had a MIC of 4 $\mu\text{g}/\text{ml}$ while the MIC for cells grown on YEMEA supplemented with the other sugars (fructose, maltose or maltodextrin) was significantly lower. This was also observed with induced cells. MIC values for rifampin were lower than erythromycin but similar trends were observed. Cells grown on induced plates with different sugars supplemented developed resistance to rifampin after 10 days (Fig. 32) while cells grown on un-induced plates did not develop resistance in the same time period. ZOI seen in Table 17 were measured at day 7.

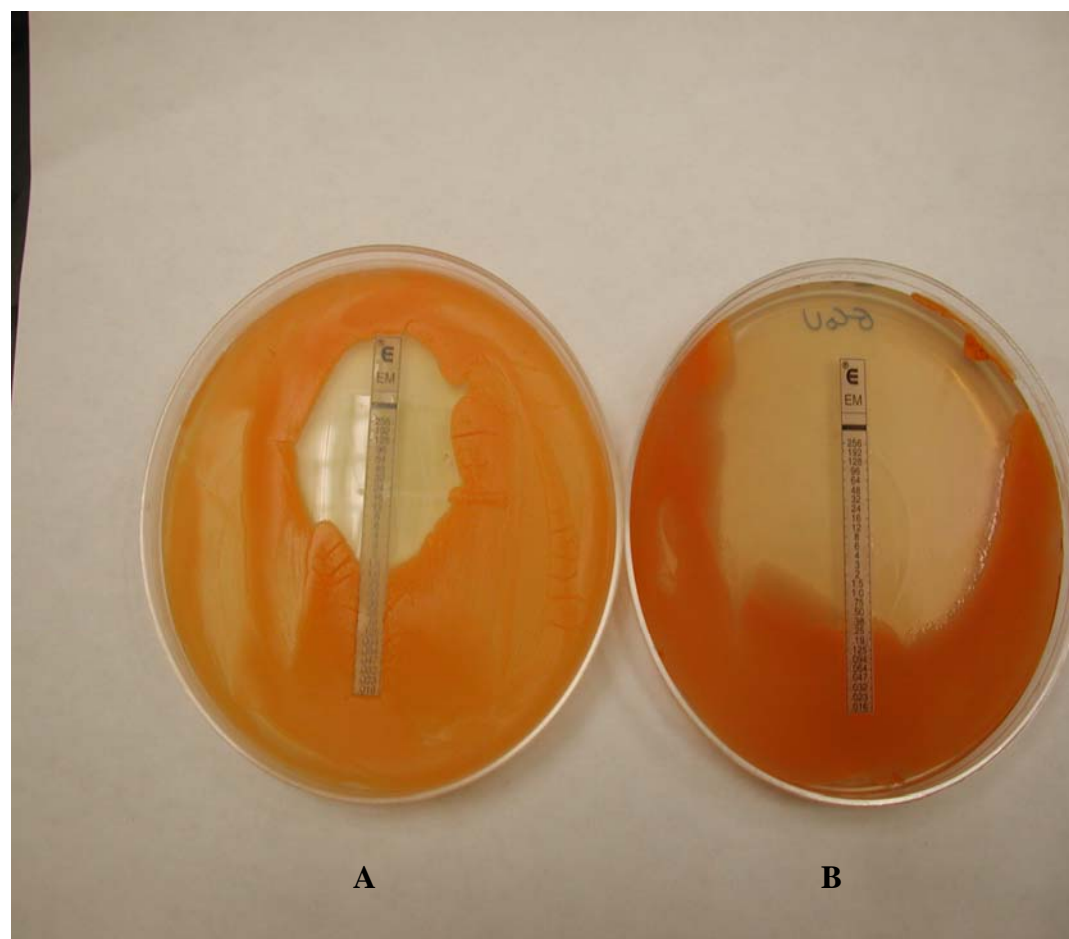


Fig. 30- MIC determination for erythromycin using Etest strip. *Rhodococcus* sp DAP 96253 grown on A) YEMEA supplemented with glucose (4g/L) B) YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L).

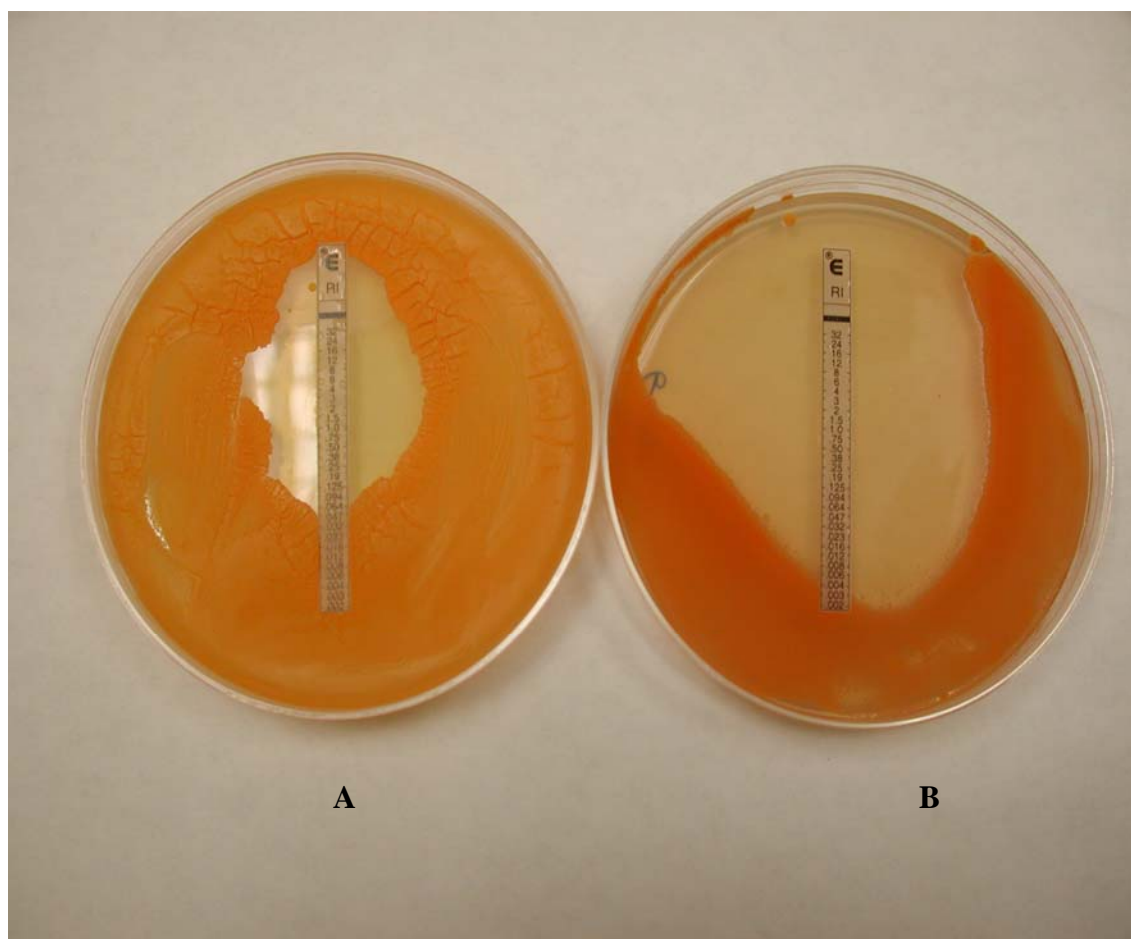


Fig. 31- MIC determination for rifampin using Etest strip. *Rhodococcus* sp DAP 96253 grown on A) YEMEA supplemented with glucose (4g/L) B) YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L).

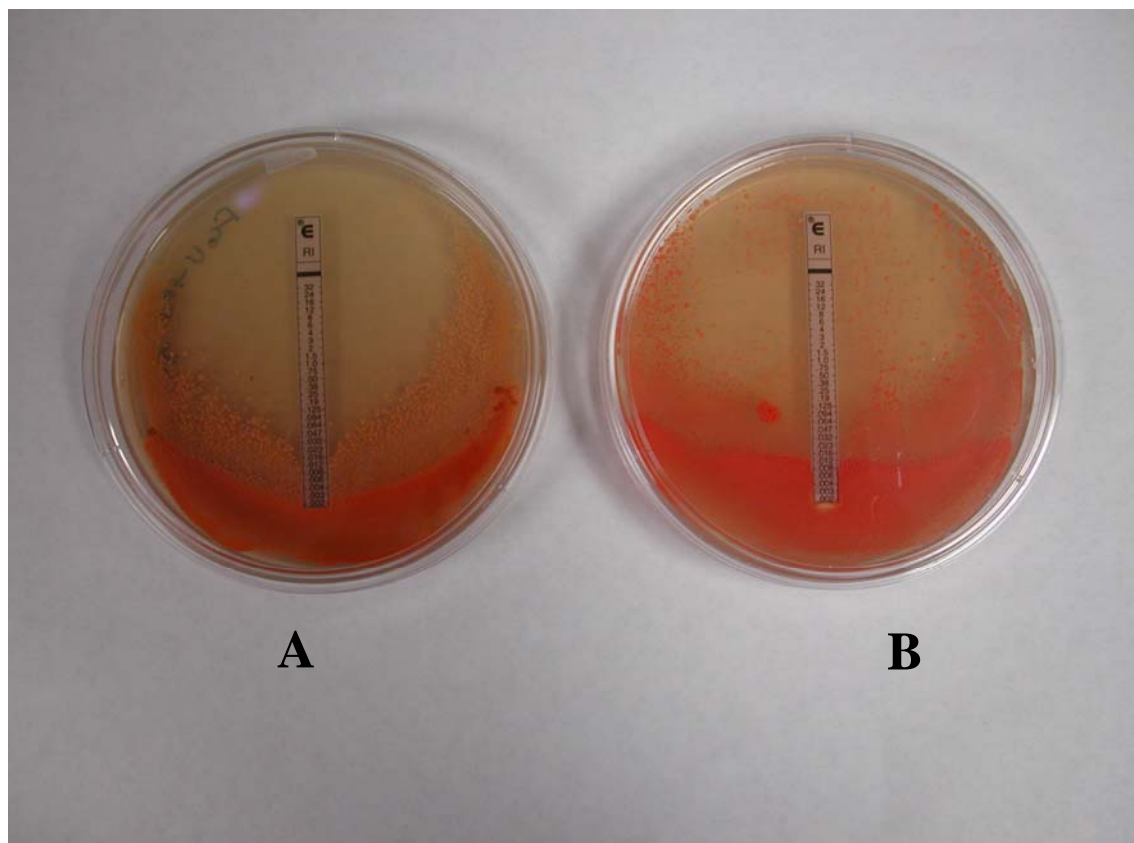


Fig. 32- Developed resistance to rifampin in *Rhodococcus* DAP 96253 after growth on YEMEA supplemented with (A) fructose (4g/L) cobalt (50mg/L) and urea (7.5g/L), (B) glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L).

Table 16. MIC ($\mu\text{g/ml}$) for erythromycin and rifampin against *Rhodococcus* sp DAP 96253

| Media | MIC of erythromycin ($\mu\text{g/ml}$) | MIC of rifampin ($\mu\text{g/ml}$) |
|-----------|------------------------------------------|--------------------------------------|
| G | 1.5 | 0.064 |
| F | 4 | 0.047 |
| M | 0.75 | 0.094 |
| MD | 2 | 0.023 |
| G, Co, U | 0.75 | 0.004 |
| F, Co, U | 0.5 | 0.004 |
| M, Co, U | 0.38 | 0.023 |
| MD, Co, U | 0.064 | 0.012 |

G- YEMEA supplemented with glucose (4g/L)

F- YEMEA supplemented with fructose (4g/L)

M- YEMEA supplemented with maltose (4g/L)

MD- YEMEA supplemented with maltodextrin (4g/L)

G, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

F, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

M, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

MD, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

MIC- Minimum inhibitory concentration

Zone of inhibition (ZOI, Fig.33 and 34, Table 17) to vancomycin and tetracycline were significantly lower when cells were grown on un-inducing YEMEA. These cells were more tolerant to the antibiotics, this was similar to the observation with erythromycin and rifampin. There were also significant differences in the ZOI when different sugars were used as supplements. Cells grown on maltodextrin supplemented YEMEA were resistant to vancomycin and tetracycline at $30\mu\text{g}$ while glucose, fructose and maltodextrin grown

cells grown cells had ZOI between 4 and 13 mm. Un-induced and induced cells were resistant to ampicillin at 10 μ g except for fructose grown cells which had a ZOI of 10 mm.

Changes in growth media composition have been shown to affect the rhodococcal cell envelope in different ways. Addition of inducers to growth media affects the cell envelope which causes the cells to become more sensitive to the antibiotics tested. Supplementation of different sugars also results in changes to the cell envelope as indicated by differences in antibiotic susceptibility.

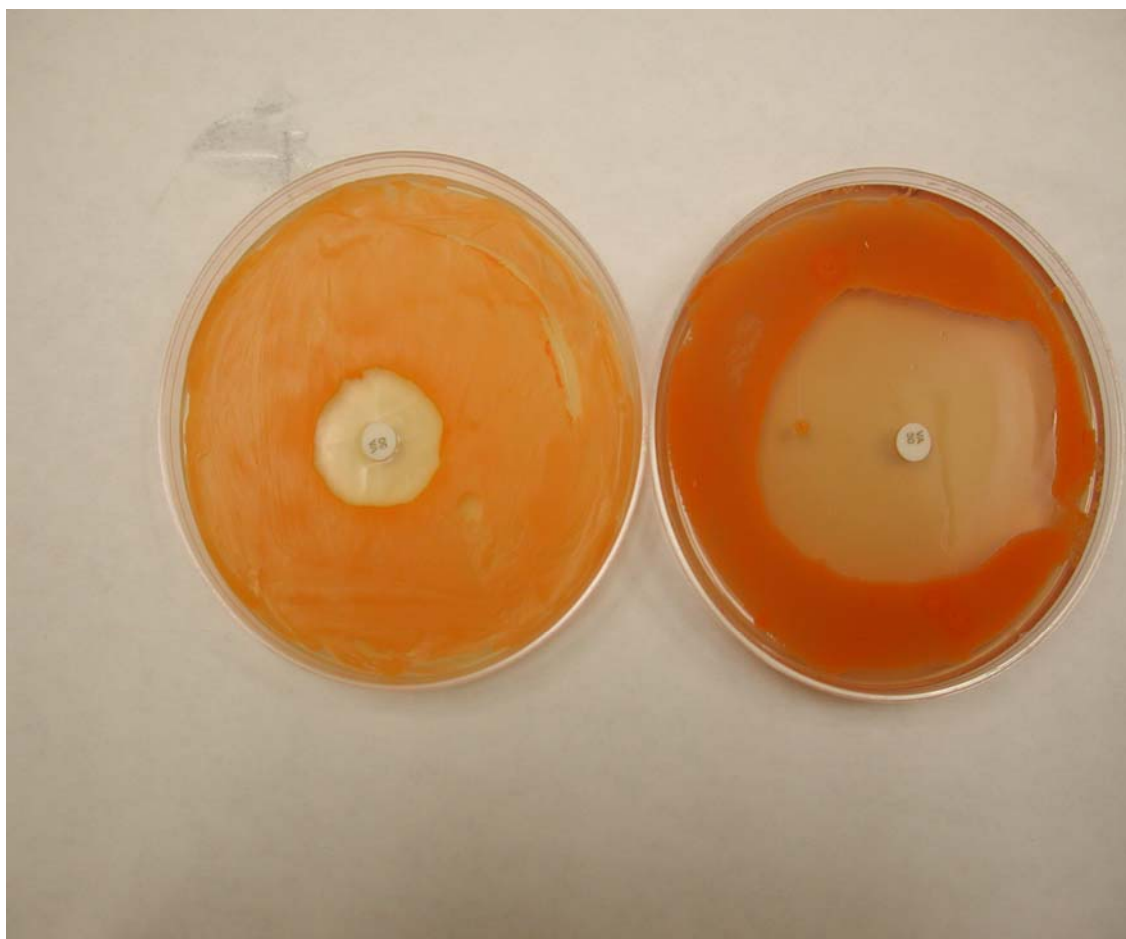


Fig. 33- ZOI for vancomycin (30µg). *Rhodococcus* sp DAP 96253 grown on A) YEMEA supplemented with fructose (4g/L) B) YEMEA supplemented with fructose (4g/L), cobalt (50mg/L) and urea (7.5g/L).

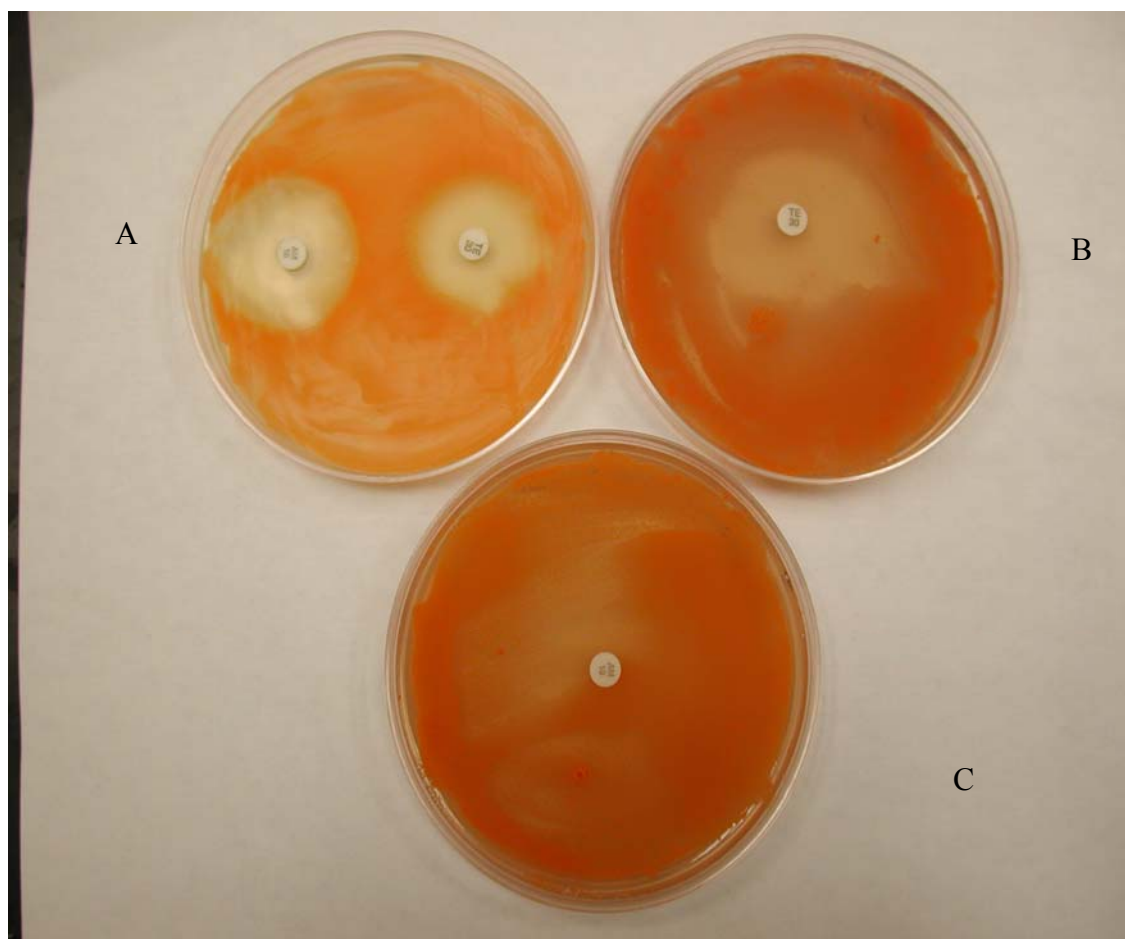


Fig. 34. ZOI for ampicillin (10 μ g), tetracycline (30 μ g). *Rhodococcus* sp DAP 96253 was grown on A) YEMEA supplemented with fructose (4g/L) with ampicillin and tetracycline sensi discs B) YEMEA supplemented with fructose (4g/L), cobalt (50mg/L) and urea (7.5g/L) with tetracycline (30 μ g) C) YEMEA supplemented with fructose (4g/L), cobalt (50mg/L) and urea (7.5g/L) with ampicillin (10 μ g)

Table 17. Zone of inhibition (mm) for vancomycin (30 μ g), ampicillin (10 μ g) and tetracycline (30 μ g) to *Rhodococcus* sp DAP 96253

| Media | Vancomycin ZOI (mm) | Ampicillin ZOI (mm) | Tetracycline ZOI (mm) |
|-----------|---------------------|---------------------|-----------------------|
| G | 10 | 0 | 4 |
| F | 8 | 10 | 10 |
| M | 13 | 0 | 13 |
| MD | 0 | 0 | 0 |
| G, Co, U | 30 | 0 | 15 |
| F, Co, U | 26 | 0 | 14 |
| M, Co, U | 30 | 0 | 17 |
| MD, Co, U | 28 | 0 | 11 |

G- YEMEA supplemented with glucose (4g/L)

F- YEMEA supplemented with fructose (4g/L)

M- YEMEA supplemented with maltose (4g/L)

MD- YEMEA supplemented with maltodextrin (4g/L)

G, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

F, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

M, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

MD, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

ZOI- zone of inhibition

The increased sensitivity of *Rhodococcus* sp DAP 96253 to antibiotics after growth on YEMEA containing cobalt and urea could be due to the presence of cobalt or urea or both cobalt and urea. This was investigated by testing the susceptibility of *Rhodococcus* sp DAP 96253 to Vancomycin Sensi-discs (30µg) and Etest strips of Erythromycin and Rifampin after growth on YEMEA supplemented with glucose only, YEMEA supplemented with glucose and urea, YEMEA supplemented with glucose and cobalt and YEMEA supplemented with glucose, cobalt and urea (Table 18). The results showed that the addition of cobalt (50 ppm) significantly increased the sensitivity of the cells to all the antibiotics used. Sensitivity to vancomycin was increased almost three times. Urea did not affect the sensitivity of the cells to the antibiotics tested.

Table 18. Susceptibility of *Rhodococcus* sp DAP 96253 to Vancomycin sensi-disc (30µg) and Erythromycin and Rifampin Etest strips.

| Media (YEMEA) | Vancomycin ZOI (mm) | Erythromycin MIC (µg/ml) | Rifampin MIC (µg/ml) |
|---------------|------------------------|-----------------------------|-------------------------|
| G | 10 | 1.5 | 0.012 |
| G, U | 10 | 1.5 | 0.032 |
| G, Co | 28 | 0.25 | 0.003 |
| G, Co, U | 26 | 0.25 | 0.004 |

G- YEMEA supplemented with glucose (4g/L)

G, U- YEMEA supplemented with glucose (4g/L) and urea (7.5g/L)

G, Co- YEMEA supplemented with glucose (4g/L) and cobalt (50mg/L)

G, Co, U- YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L)

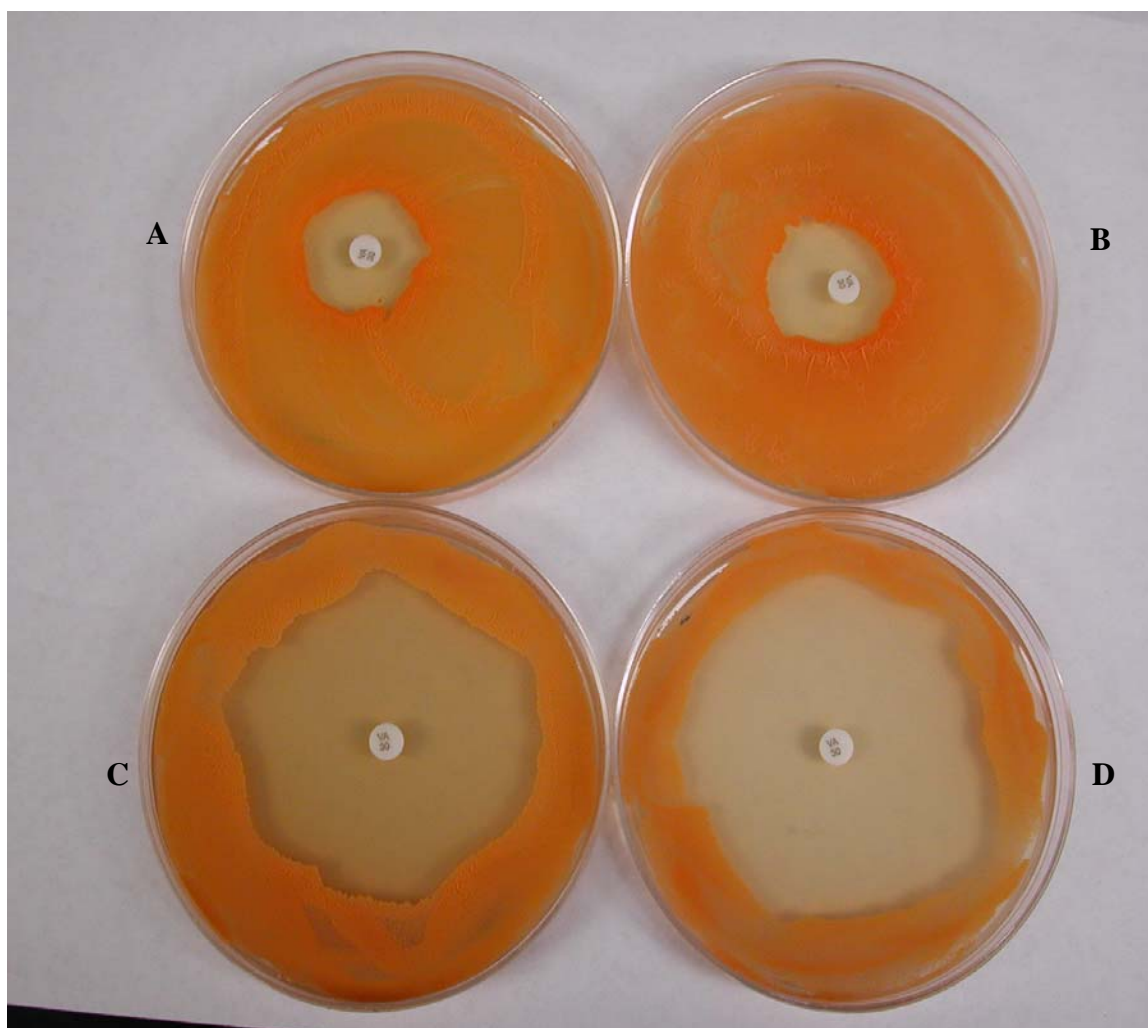


Fig. 35- *Rhodococcus* sp DAP 96253 susceptibility to vancomycin (30µg) after growth on YEMEA with different supplements. A) YEMEA supplemented with glucose (4g/L) B) YEMEA supplemented with glucose (4g/L) and urea (7.5g/L) C) YEMEA supplemented with glucose (4g/L) and cobalt (50mg/L) D) YEMEA supplemented with glucose (4g/L), cobalt (50mg/L) and urea (7.5g/L).

Discussion

Rhodococcus sp DAP 96253 was chosen as the selected strain to carry out these investigations due to its high NHase activity on un-inducing and inducing YEMEA compared to other rhodococcal species investigated. YEMEA is composed of yeast extract and malt extract both which have the primary carbon source, glucose. There was supplementation of glucose to YEMEA due to increased growth and increased NHase activity. Other sugars such as fructose (a monosaccharide), maltose (a disaccharide) and maltodextrin (a polymer of glucose, 25 DE) also were used as supplements in YEMEA to examine effects on NHase activity. Wang et al. (2006) reported that supplementation of glycerol in a complex medium for glucose and sucrose resulted in slightly slower growth rate and a significant increase in NHase production, these findings supported the results in this dissertation as supplementation of fructose, maltose or maltodextrin for glucose in rich solid media (YEMEA) also resulted in slight differences in growth rate and significant differences in NHase activity.

Propionitrile (PN) was the best substrate for the NHase from *Rhodococcus* sp DAP 96253, followed by acrylonitrile (AN), butyronitrile (BN) and crotonitrile (CrN). There was a 32% difference in NHase activity between the substrates PN and AN when maltose was supplemented into YEMEA with cobalt and urea and a 26% difference in NHase activity when the cells were grown on YEMEA supplemented with maltodextrin, cobalt and urea (Table 6). The chemical formulas CH_2CHCN , $\text{CH}_3\text{CH}_2\text{CN}$, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CN}$, CH_3CHCHCN represent AN, PN, BN and CrN

respectively. AN and PN have three carbon atoms, these two compounds are very similar in structure except for the double bond in AN between carbon 1 and 2. CrN and BN have four carbon atoms, they are also similar in structure but CrN has a double bond between carbons 2 and 3. NHase works best for short chain nitriles with no double bonds like PN. Growth of *Rhodococcus* sp DAP 96253 on YEMEA supplemented with different carbohydrates resulted in statistically significant differences in NHase activity against AN and PN (Table 7 and 8). This shows that supplementation of different sugars or carbohydrates such as maltodextrin influences NHase activity therefore growth media selection can play a role in substrate specificity. The same cell can be tailored for different reactions by changing the carbohydrate supplementation in the growth media.

Rhodococcus sp DAP 96253 can be grown in the presence of different sugars to facilitate reactions at different temperatures. The production of acrylamide is done at low temperatures in order to minimize conversion of acrylamide to acrylic acid by amidase (Nagasawa et al., 1993; Padmakumar and Oriel, 1999). Growth of *Rhodococcus* sp DAP 96253 on YEMEA supplemented with maltose or maltodextrin with cobalt and urea, had higher NHase activity at 10°C than cells grown on fructose or glucose. This data can be applied in industries that employ NHase at lower temperatures such as acrylamide production.

Supplementation of growth media with different primary sugars not only affected the NHase activity but also affected the stability of the enzyme. NHase in cells of

Rhodococcus sp DAP 96253 grown on maltose and maltodextrin was statistically more stable at 55°C than NHase in glucose or fructose grown cells. This was possibly due to differences in cellular trehalose levels within cells. Trehalose can be synthesized from glucose, maltose and maltodextrins in *Corynebacterium* through three pathways seen in Fig. 4. There are no reports that *Rhodococcus* possesses these three pathways, but growth on YEMEA supplemented with glucose, fructose, maltose or maltodextrin with and without cobalt and urea, resulted in changes in both cellular trehalose levels and lipid bound trehalose levels (Table 13 and 14).

There have been reports on intracellular changes of trehalose content in bacteria and fungi. However, these changes have been induced by stress such as extremes in temperatures or exposure to chemicals or organic solvents (Joo et al., 2000; Tibbett et al., 2002). This work shows that significant changes in cellular trehalose levels can occur without stress on the cell. The addition of different carbohydrates and inducers (cobalt, urea and ASN) of NHase not only affected the NHase activity, but also influenced the cellular and lipid bound trehalose levels.

Xie and Timasheff (1997) showed that when 0.5M trehalose was added to purified enzyme stability was enhanced. In this work improvement to NHase stability at 55°C was achieved through the addition of trehalose to the growth media. Addition of trehalose to YEMEA increased thermostability of NHase in cells grown on YEMEA supplemented with glucose or fructose, cobalt and urea (Table 11). The three-dimensional structure of proteins is maintained by polyols such as sugars during

heating and drying. They act as protein stabilizers by forming hydrogen bonds with the protein in place of water (Arakawa, 2002). The supplementation of different sugars in YEMEA resulted in varying levels of trehalose within the cells, that acted as a stabilizer of NHase, this accounted for differences observed in NHase stability at 10°C and 55°C. The addition of trehalose to YEMEA supplemented with maltose or maltodextrin did not achieve any additional enhancement of NHase stability. Cui et al. (2006) showed that maltodextrin was able to stabilize transglutaminase from *Streptomyces hygroscopicus* better than trehalose. It is possible that maltose and maltodextrin also provided some stabilizing effect in addition to trehalose that led to the increase stability of the NHase.

Glutaraldehyde immobilization has been shown previously to be quite effective in stabilizing NHase in rhodococcal cells. However during the immobilization process a significant amount of NHase activity can be lost (20-60%, Ganguly, Dissertation). This work showed that NHase activity and stability of native cells grown on YEMEA supplemented with maltose or maltodextrin, cobalt and urea were significantly higher than cells grown on YEMEA supplemented with glucose, cobalt and urea. Changing the carbohydrate constituents in YEMEA might result in production of catalysts that retain more NHase activity. The data showed that supplementation of different sugars in the growth media affects cellular trehalose levels, this can impact the stability of NHase.

Changes in the carbohydrate supplement and the addition of inducers for NHase to YEMEA resulted in demonstrable changes in the cell envelope of *Rhodococcus* sp DAP 96253. Numerous researchers have shown that growth media can influence mycolic acid. Previously changes in HPLC profiles of mycolic acids were observed when hydrocarbons replaced glucose as the carbon source in growth media, or when hydrocarbons of various chain lengths were used (Sokolovska et al., 2003). However, analysis of the HPLC profiles of mycolic acids showed that supplementing fructose, maltose, or maltodextrin for glucose in YEMEA resulted in significant changes, therefore changes in carbon source does not have to be drastic as replacing a sugar for a hydrocarbon to see differences in mycolic acid profiles. The addition of NHase inducers such as cobalt and urea also resulted in changes in mycolic acid profiles suggesting that other components of growth media, such as metals and amides can have an impact on mycolic acids content of *Rhodococcus* sp DAP 96253.

The examination of mycolic acid profiles is a powerful and relatively easy tool that has been used by the Center for Disease Control (CDC) in species identification of *Mycobacterium*, in differentiating *Rhodococcus* species and in distinguishing members of the mycolata taxon (CDC, 1996; Butler et al., 1986). Media selection would be extremely important when mycolic acid profiles are being used in the above applications as changes in sugars and metals in the media can affect mycolic acid profiles, which could lead to problems in identification, screening and classification of the mycolata.

Tropis et al. (2005) reported that a *Corynebacterium glutamicum* mutant defective in trehalose biosynthesis was unable to synthesize mycolic acids outside the cell. However, the addition of glucose, maltose, or maltotriose to the growth media of the trehalose defective mutant *C. glutamicum* restored mycolic acid synthesis by restoring trehalose synthesis. Gebhardt et al. (2007) 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. 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. These changes in cellular trehalose may have contributed to the changes seen in mycolic acid content and composition in the cell envelope of *Rhodococcus* sp DAP 96253.

Susceptibility of *Rhodococcus* sp DAP 96253 to Erythromycin, Rifampin, Vancomycin, Ampicillin and Tetracycline was examined. The investigation showed that addition of cobalt and urea led to increased sensitivity to all the antibiotics tested except for Ampicillin (10µg). In addition, changes in carbohydrate supplements in YEMEA resulted in differences in minimum inhibitory concentrations (MICs) and zone of inhibitions (ZOIs). These results further supported the hypotheses that changes were occurring in the cell envelope of *Rhodococcus* sp. DAP 96253.

Etest[®] was used to for Erythromycin and Rifampicin. Both antibiotics target bacterial protein synthesis (Reese et al., 1994). *Rhodococcus* sp DAP 96253 was more susceptible to Rifampin than Erythromycin. Cells of *Rhodococcus* sp. DAP 96253 induced for NHase and then exposed to the Rifampin later developed resistance to rifampin. Delayed resistance to Rifampin did not occur with un-induced cells. Rifampin is used mostly with mycobacteria, which are known to develop resistance to this particular antibiotic (Inderlied, 1991). Resistance could be linked to cobalt acquisition in the media since growth in the presence of cobalt and urea led to resistance in *Rhodococcus* sp DAP 96253.

Vancomycin, which inhibits cell wall synthesis in Gram-positive bacteria (Reese et al., 1994), was the most effective antibiotic used in the ZOI experiment. *Rhodococcus* sp DAP 96253 cells induced for NHase activity with cobalt, urea and glucose supplementation were approximately three times more sensitive to Vancomycin than cells un-induced for NHase (Table 16). Cells grown on YEMEA supplemented with fructose and maltodextrin were not as susceptible to Vancomycin as glucose and maltose grown cells. Maltodextrin induced cells were resistant to Vancomycin.

Ampicillin is an inhibitor of cell wall synthesis (Reese et al., 1994). *Rhodococcus* sp DAP 96253 grown on YEMEA with glucose, maltose or maltodextrin with and without cobalt and urea were resistant to Ampicillin. Only cells grown on YEMEA with fructose un-induced for NHase showed sensitivity to Ampicillin (10µg). This

might indicate that growth on fructose resulted in changes in the cell envelope that allowed the antibiotic easier access to the cells.

Sensitivity to antibiotics was increased significantly in cells induced for NHase, this increased sensitivity might be due to the changes occurring in the cell envelope of *Rhodococcus* sp DAP 96253. The results showed that the addition of cobalt, and sugars to the media affected the mycolic acid profiles, this indicated changes in the cell envelope. The addition of cobalt to growth media can affect other components of the cell besides the cell envelope. Guymon et al (1978) showed that increased resistance to multiple antibiotics in *Neisseria gonorrhoeae* was due to changes in the cell envelope accompanied by changes in outer membrane proteins. Increased sensitivity in induced cells of *Rhodococcus* sp DAP 96253 could be due to changes in mycolic acids and glycolipids possibly accompanied by the presence of outer membrane proteins induced by the presence of cobalt. Increased resistance to rifampin was also observed in induced rhodococcal cells this could also be due to the presence of outer membrane proteins induced by the presence of cobalt.

Cobalt was shown to play a role in antibiotic susceptibility in *Rhodococcus*. This metal might also influence antibiotic susceptibility in other organisms within the mycolata taxon such as *Mycobacterium* and *Corynebacterium*. *Rhodococcus*, *Mycobacterium* and *Corynebacterium* have species that are pathogenic, using cobalt in conjunction with antibiotics could be a treatment option. *Mycobacterium* infections are generally harder to treat, utilizing cobalt in treatment might make the cells more

susceptible to antibiotics and result in shorter treatments, or the use of lower doses of toxic antibiotics.

Overall the three hypotheses made in the rationale were supported. NHase activity was affected by changes in media composition. Supplementation of fructose, maltose, maltodextrin in growth media resulted in increased NHase activity. NHase stability was also affected by supplementation of different sugars, this could be related to changes in cellular and trehalose levels. The supplementation of different sugars and inducers of NHase elicited changes in cell envelope components such as mycolic acids and glycolipids.

Conclusions

Supplementation of different carbohydrates and cobalt in YEMEA resulted in changes in the cell envelope of *Rhodococcus* sp DAP 96253. Changes in the cell envelope were observed by HPLC profiles of mycolic acids, by TLC of lipid extracts, and by differences in antibiotic susceptibility.

Differences in NHase activity after *Rhodococcus* sp DAP 96253 was grown on YEMEA with different carbohydrate supplementations and inducers of NHase can be attributed to changes in cell envelope components such as mycolic acids, glycolipids and trehalose.

Trehalose is a component of the cell and cell envelope had a significant impact on NHase stability. This information can be used in the preparation of whole cells as immobilized catalysts. Trehalose can be added to growth media or be used as storage solution for cells to maintain stability of NHase before and after the immobilization process.

Immobilization of rhodococcal whole cells that are stable with high activity is important in large scale treatment of waste or in the production of acrylamide. Any factor that affects the cell envelope such as growth media or culture conditions will have an effect on immobilization process as these reagents have to enter the cell, and crosslink proteins. There is loss of enzyme activity in the immobilization of *Rhodococcus*, therefore it is imperative that whole cells used in any immobilization process have high enzyme activity and are inherently stable, this can be achieved by

Careful media selection. Media components and culture conditions can be manipulated to create a cell whose cell envelope would allow enzymes such as NHase to function at elevated or lower temperatures, also cells could be tailored for increased tolerance to substrates and products. The stabilization of the native cell through manipulation of its own metabolism without the addition of foreign/toxic chemicals is advantageous as more stable cells can be produced cheaper and safer.

The isolation of enzymes from cells is an expensive process which usually results in significant loss of enzyme activity and stability. The use of whole cell catalysts is far more practical for industrial applications. This work provides significant background on how the entire cell impacts enzyme activity and stability. Improvement in NHase activity and stability is dependent upon on the components of the cell wall and other key metabolites of the cell such as trehalose.

Future Directions

Additional research is needed in order to identify specific changes occurring in mycolic acids, and to identify glycolipids produced after growth on YEMEA with different supplementations. The presence of other sugars in the cell and cell wall should be evaluated. Also investigations on the effect of immobilization with glutaraldehyde and other immobilization agents on the loss of NHase activity after growth on YEMEA supplemented with different sugar and inducers would be informative. Rhodococcal trehalose mutants would provide additional information on the role of trehalose in NHase activity, stability and cell envelope permeability. The effect of cobalt on antibiotic sensitivity in *Rhodococcus* and other members of mycolata should also be explored.

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Appendix

Ammonia Assay

Sodium phenate solution

25 g Phenol (Sigma- Aldrich Co., St. Louis, MO) added to 800 ml water and 78 ml 4N sodium hydroxide (Sigma- Aldrich Co., St. Louis, MO) solution.

Sodium Nitroprusside Solution (0.1%)

1 g of Sodium Nitroprusside (Sigma- Aldrich Co., St Louis, MO) added to 100 ml DI water (stock solution).

1 ml of stock solution was added to 99ml DI water.

Sodium hypochlorite (0.02N)

2.44 ml of 6.15% Chlorox[®] made up to 100 ml with DI water

Mycolic acid extraction

Saponification reagent

200 g of potassium hydroxide (Sigma, St. Louis, MO) was added to 400 ml DI water followed by the addition of 400 ml of methanol

Acidification reagent

50% solution of hydrochloric acid (Fisher Scientific, Fairlawn, NJ).

Potassium bicarbonate reagent

4 g of potassium bicarbonate (Sigma, St. Louis, MO) was added to 98 ml of DI water and 98 ml of methanol (Sigma-Aldrich, St. Louis, MO).

Clarification Reagent

100 ml of the acidification reagent added to 100 ml of methanol (Sigma-Aldrich, St. Louis, MO).

SDS- PAGE Gel

12% T (Bottom) Gel

20.8 ml of Protogel (30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide stock solution, National Diagnostics, Atlanta, GA) was added to 16 ml ddH₂O, 12.5 ml of 1.5M Tris (Sigma, St. Louis, MO) pH 8.8, and 500 µl 10% SDS (GE Healthcare Biosciences, Uppsala, Sweden). The solution was de-aerated for 10 min followed by the addition of 25 µl TEMED (Sigma, St Louis, MO).

4% T Stacking (Top) Gel

2.6 ml of Protogel (30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide stock solution, National Diagnostics, Atlanta, GA) was added to 12.12 ml ddH₂O, 5 ml of 1.5M Tris (Sigma, St. Louis, MO) pH 6.8, and 200 ul 10% SDS (GE Healthcare Biosciences, Uppsala, Sweden). The solution was de-aerated for 5 min followed by the addition of 10 µl TEMED (Sigma, St Louis, MO).

Protein Staining, Digestion and Zip Tipping

Silver Staining Reagents

Fixing Solution

250 ml DI water added to 200 ml ethanol (Sigma-Aldrich, St Louis, MO) and 50 ml acetic acid (Fisher Scientific, Fairlawn, New Jersey)

Sensitizing solution

200 ml DI water added to 10 ml 5% sodium thiosulfate (Fluka Chemie, Buchs, Switzerland) solution with 17 g sodium acetate (Sigma, St Louis, MO) made up to 250 ml with DI water, 1.25 ml of glutaraldehyde (Fluka Chemie, Buchs, Switzerland) added immediately before use.

Silver solution

250 DI water added to 625 mg silver nitrate (Sigma, St Louis, MO), 100 μ l of formaldehyde (Sigma, St Louis, MO) added immediately before use.

Developing solution

7 μ l 5% sodium thiosulfate (Fluka Chemie, Buchs, Switzerland) and 6.25 g sodium carbonate (Sigma, St Louis, MO) added to 200 ml DI water and made up to 250 ml, 100 μ l of formaldehyde (Sigma, St Louis, MO) added immediately before use.

Stopping solution

3.65 g EDTA (Fisher Scientific, Fairlawn, New Jersey) added to 250ml of DI water

Preserving solution

150 ml ethanol (Sigma, St Louis, MO) added to 327 ml of DI water with 23 ml of glycerol (Sigma, St Louis, MO).

Gels for silver staining were placed in the fixing solution for two 15 min periods then placed in the sensitizing solution for 30 min. The gels were washed three times for 5 min, then stained with silver for 20 min, followed by washing twice for 1 min. Bands were developed in the developing solution for 4-10 min and stopped by placing the gel in the stopping solution for 10 min. Gels were washed three times for 5 min in DI water then placed in the preserving solution.

Hot Coomassie Blue Staining

1.6 L of gel staining solution consisting of 1 PhastGel® (Amersham Biosciences, Piscataway, NJ) tablet dissolved in 10% acetic acid (Fisher Scientific, Fairlawn, NJ) was heated and poured over the gels. The gel was incubated in the staining solution for 10 min. The gel was transferred to a separate tray and destained three times for 1 hr in 1.1 L of 10 % acetic acid with gentle agitation. Gels were transferred to 500 ml of a preserving solution (87% glycerol, GE Healthcare Biosciences, Uppsala, Sweden) for 30 min.

Protein Digestion

Gel plugs were incubated for 20 min at room temperature in 100 μ l of 50mM ammonium bicarbonate (Sigma, St. Louis, MO) in 50% acetonitrile (Applied Biosystems, Warrington, UK) the solution was removed and the step was repeated, 100 μ l of 75% acetonitrile (Applied Biosystems, Warrington, UK) was added to the plugs and incubated for 20 min at room temperature. The plugs were dried using a speed vacuum for 30 min with no heat. The protein was digested by adding 7 μ l of 20 μ g/ml trypsin (Sigma, St. Louis, MO) to the plugs and incubating them at 37°C overnight. The digested proteins were incubated for 20 mins with 60 μ l of 50% acetonitrile/0.1% TFA, following incubation the solution was transferred into a new tube and an additional 40 μ l of a 50% acetonitrile (Applied Biosystems, Warrington, UK) with 0.1% TFA (Applied Biosystems, Warrington, UK) solution was added to the plugs and incubated again for 20 mins. The solution was removed and added to the tube containing the first extract, then dried in speed vacuum with no heat for 5hrs.

Zip Tip (uC18) for MALDI TOF/TOF

Dried samples from protein digestion were treated with neat formic acid (Sigma Aldrich, St. Louis, MO) and vortexed, 8.5 μ l of 0.1% TFA (Applied Biosystems, Warrington, UK) was added to the tube and the solution vortexed. Zip tips were prepared by aspirating the wetting solution (50% acetonitrile, Applied Biosystems, Warrington, UK) twice followed by aspirating and discarding 0.1% TFA ten times. The

sample was bound to the Zip Tip by aspirating and dispensing the sample inside the sample Eppendorf tube ten times. The sample was washed in 0.1% TFA ten times. A second pipette was used to dispense 0.7 μ l of elution solution (0.1% TFA / 70% acetonitrile) into the cap of the sample Eppendorf tube. The entire droplet was aspirated and eluted five times into the cap of the Eppendorf tube and on the final rinse the entire volume of the sample was aspirated and dispensed onto a desired space on a clean MALDI plate. The sample was allowed to dry and 0.3 μ l of alpha matrix (Applied Biosystems, Warrington, UK) added onto the sample spot.

Trehalose analysis

Anthrone reagent

200 mg of anthrone (Fisher Scientific, Fairlawn, NJ) in 5 ml absolute ethanol (Aaper Alcohol and Chemical Co., Shelbyville, KY) made up to 100 ml with 75% sulfuric acid (Fisher Scientific, Fairlawn, NJ).