Analysis of dextrin dextranase from *Gluconobacter oxydans.*

by

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Declaration

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Abstract

Dextran is a high value glucose polymer used in medicine and an array of laboratory techniques. It is synthesised by lactic-acid bacteria from sucrose but has also reportedly been produced by *Gluconobacter oxydans* (*G. oxydans*) from a range of maltooligosaccharides (MOS) via the action of dextrin dextranase (DDase). In this study the presence of DDase is investigated in two *G. oxydans* strains (ATCC 621H and ATCC 19357) and shown to be present in the ATCC 19357 strain, but not in the ATCC 621H strain. The enzyme was partially purified from the ATCC 19357 strain, and its kinetic properties investigated. The partially purified protein was also digested with trypsin, and *de novo* peptide sequences obtained from it. Several attempts were made to obtain the gene coding for the DDase. These include amplifying an open reading frame from the *G. oxydans* genome coding for a glycosyltransferase with the approximate molecular weight of the DDase, using the peptide sequences obtained from the partially purified protein to design degenerate PCR primers and the production of a genomic DNA library for functional screening in *E. coli*. None of these approaches led to the successful isolation of the extracellular DDase sequence.

Abstrak

Dextraan is 'n hoë waarde glukose polimeer wat veral van belang is in die mediese praktyk sowel as vir 'n aantal laboratorium tegnieke. Dit word hoofsaaklik deur melksuurbakterië vanaf sukrose gesinteseer maar kan ook deur *Gluconobacter oxydans* (*G. oxydans*) vervaadig word deur die werking van dextrin dextranase (DDase) op 'n reeks maltooligosakkariede (MOS). In hierdie studie is die teenwoordigheid van DDase in twee *G. oxydans* rasse (ATCC 621H en ATCC 19357) ondersoek. Daar is bepaal dat die ensiem teenwoordig is in die ATCC 19357 ras, maar nie in die ATCC 621H ras nie. Die DDase ensiem is gedeeltelik gesuiwer vanuit die ATCC 19357 ras, en die kinetiese eienskappe daarvan is ondersoek. Die gedeeltelik gesuiwerde proteïen is met trypsien verteer, en die *de novo* peptiedvolgorde is vasgestel. Verskeie pogings om die volgorde van die DDase geen te verkry is aangewend. Hierdie pogings sluit in die amplifikasie van 'n oop leesraam van die *G. oxydans* genoom wat kodeer vir 'n glikosieltransferase met ongeveer dieselfde molekulêre massa as die van DDase, die gebruik van die bepaalde peptied volgordes van die gedeeltelik gesuiwerde ensiem om degenereerde PKR inleiers te ontwerp, asook die produksie van 'n genoomiese DNS biblioteek vir funktioneele skerming in *E. coli*. Geen van hierdie benaderings het gelei tot ‘n suksesvolle bepaaling van die ektrasellulêre DDase geenvolgorde nie.

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‘Rage, rage against the dying of the light’ – Dylan Thomas

Table of Contents

pg

Declaration 2

Abstract 3

Acknowledgements 4

List of Figures 8

List of Tables 9

List of Abbreviations 10

Chapter 1

pg

1.1 Introduction 12

1.2 DDase mode of action 12

1.3 Substrate specificity 14

1.4 DDase isozymes 17

1.5 Temperature and pH optima 18

1.6 Inhibitors and activators 19

1.7 Glycosyltransferases from the *G. oxydans* genome 19

1.8 Applications of *G. oxydans* dextran 20

References 21

Chapter 2

Section 1

Introduction 24

References 27

Section 2

2.2.1 Materials 29

2.2.2 Methods 29

2.2.2.1 Extraction of proteins from cell-free culture 29
2.2.2.2 SDS-PAGE and activity gel analysis 30
2.2.2.3 Agarose gel DNA electrophoresis 30
2.2.2.4 Cloning of putative DDase gene into pGEX-4T-1 30
2.2.2.4.1 PCR of putative gene 30
2.2.2.4.2 Ligation of PCR product into pGEM-T Easy 31
2.2.2.4.3 Plasmid purification 31
2.2.2.4.4 Restriction analysis and ligations 31
2.2.2.4.5 Production and partial purification of GST-fusion proteins 32
2.2.2.5 Immunoblots 32
2.2.2.6 Functional analysis of genes in the *E.coli pgm* mutant 33
2.2.2.7 Kinetic parameter of the enzyme using maltotriose as a substrate 33
2.2.2.8 Assay using ρ-Nitrophenyl-α-D-glucopyranoside (NPG) as a substrate 34
2.2.2.9 Dextran production 34
2.2.2.10 Glucose content of hydrolysed dextran samples 34
2.2.2.11 Assay for free glucose 35
2.2.2.12 Activity with GST-fusion protein 35
2.2.2.13 Digestion of dextran with dextranase 35
2.2.2.13.1 Digestion of dextran with dextranase *in vitro* 35
2.2.2.13.2 Digestion of dextran with dextranase in gel 36
2.2.2.14 Digestion of isomaltose with α-Glucosidase (Type I) 36
2.2.2.15 Analysis by Thin Layer Chromatography (TLC) 36
2.2.2.16 Schiff staining 36
2.2.2.17 Genomic DNA extraction 37
2.2.2.18 Tryptic digest 37
2.2.2.19 Peptide sequencing of DDase from *Gluconobacter oxydans* ATCC 19357 38
2.2.2.20 PCR of degenerate primers designed from peptide sequences 39
2.2.2.21 Construction of the *G. oxydans* genomic library 40
2.2.2.22 Bradford protein determinations 40
2.2.2.23 Sequencing 40

References 41

Chapter 3

Results and discussion 42

References 61

Chapter 4

Conclusion 62

List of Figures

Chapter 1 pg

Figure 1. Summary of the three modes of action of DDase. 15

Figure 2. Graph of K_m values for several MOS and short chain amylose with increasing DP values. 17

Chapter 3 pg

Figure 1. LBA plate with *E. coli pgm* mutants transformed with control pBluescriptSK and pBluescriptSK with *GOX0496* sequence. 43

Figure 2. A 1% (w/v) agarose gel with DNA molecular weight ladder, negative template control and the PCR amplicon. 44

Figure 3. A 10% SDS-PAGE with molecular weight markers and four elution fractions. 45

Figure 4. Immunoblot of GST tag and GST/ GOX0496 fusion proteins. 45

Figure 5. The activities of the GST/GOX0496 fusion protein and the GST tag. 46

Figure 6. A 6% native gel with protein from cell-free cultures of ATCC 19357 and 621H. 48

Figure 7. Two 8% activity gels incubated with MOS. 49

Figure 8. A 10% (v/v) SDS-PAGE of the extracellular proteins from *G. oxydans* ATCC 19357 and *G. oxydans* ATCC 621H. 50

Figure 9. Production of glucose from maltotriose for two strains of *Gluconobacter oxydans,* ATCC 621H and ATCC 19357. 51

Figure 10. TLC of hydrolysis products of dextran produced from partially purified protein from *G. oxydans* strains ATCC 19357 and ATCC 621H. 52

Figure 11. TLC of digestion products of dextran from *L. mesenteroides* and dextran produced from DDase using maltotriose and MOS and enzymatic digestions thereof. 53

Figure 12. Graph of DDase activity. 55

Figure 13. Four sets of degenerate primers with were used to amplify regions of *G. oxydans* ATCC 19357 gDNA. 57

Figure 14. Four sets of degenerate primers with were used to amplify regions of *G. oxydans* ATCC 19357 gDNA. 57

Figure 15. Partial digestion of *G. oxydans* ATCC 19357 gDNA. 58

Figure 16. DNA fragments digested with *Sau3A1*. 59

List of Tables

Chapter 1 pg

Table 1. Donor and acceptor specificity of intracellular DDase. 13

Table 2. Dextran yield when DDase is incubated with various substrates. 16

Table 3. Glycosyltransferases and putative glycosytransferases from internet genome sources. 20

Chapter 3 pg

Table 1. Comparisons of experimental results. 48

Table 2. Peptide sequence matches from a tryptic digest. 55

Table 3. Sequence analysis results of seven amplicons amplified using gDNA from *G. oxydans* ATCC 19357. 56

Table 4. Ten colonies of *pgm* mutants which stained lightly with iodine. 60

List of Abbreviations

(w/v)	Weight per volume
(w/w)	Weight per weight
bp	Base pairs
BSA	Bovine serum albumin
CDS	Conserved Domain Sequence
DDase	Dextrin dextranase
$DDase_{ext}$	Extracellular DDase
$DDase_{int}$	Intracellular DDase
ddH_2O	Distilled and deionised water
DNA	Deoxyribonucleic acid
DP	Degree of polymerisation
DSase	Dextransucrase
DSMZ	German Resource Centre for Biological Material Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
DTT	Dithiotreitol
EDTA	Ethylenediaminetetra-acetate
EtOH	Ethanol
G. oxydans	*Gluconobacter oxydans*
gDNA	Genomic DNA
GBSS	Granule Bound Starch Synthase
GST	Glutathione S-transferase
HCl	Hydrochloric acid
HPLC	High Pressure Liquid Chromatography
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IPTG	Isopropyl β-D-1-thiogalactopyranoside
isoMOS	Isomaltooligosaccharides
Kb	Kilo bases
kDa	kilo Dalton
KOH	Potassium hydroxide
LB	Luria broth

LBA	Luria broth agar
LC-MS-MS	Liquid Chromatography/ Mass Spectrometry/ Mass Spectrometry
m/z	mass/ charge
MOS	Maltooligosaccharides
MW	Molecular weight
NBT/BCIP	Nitro blue tetrazolium/ 5-Bromo-4-chloro-3-indolyl phosphate
NCBI	National Center for Biotechnology Information
NPG	ρ-Nitrophenyl-α-D-glucopyranoside
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
ρCMB	ρ-Chloromercuribenzoic acid
PCR	Polymerase Chain Reaction
PFU	Plaque forming unit
PGM	Phosphoglucomutase
PVDF	Polyvinylidene fluoride
RE	Restriction endonuclease
SDS	Sodium dodecyl sulphate
TLC	Thin Layer Chromatography
X-gal	beta-D-galactopyranoside

Chapter 1

Introduction

Strains of acetic acid bacteria identified as *Acetobacter viscosum* (Baker *et al*., 1912) and *Acetobacter capsulatum* (Shimwell, 1947) are shown to be associated with a type of beer spoilage known as 'ropiness'. These species have since been reassigned to the *Gluconobacter* genus (De Ley and Frateur, 1970). Ropiness is ostensibly due to bacterial production of capsular material from dextrins, natural constituents of beer. Viscousity develops when *A. viscosum* and *A. capsulatum* are cultured in dextrin rich beers or in yeast extract media containing dextrin. Ropiness is not observed in beer low in dextrins, nor if dextrins in the yeast extract media is replaced with another carbon source such as glucose, fructose, maltose or sucrose. The capsular material consists primarily of maltooligosaccharides (MOS) and dextran (Shimwell, 1947). Dextran isolated from spoiled beer is shown to be immunologically similar to dextran synthesized by *Leuconostoc mesenteroides* via dextransucrase (DSase; Hehre and Hamilton, 1949). It was later shown that a partially purified extracellular enzyme produced by *A. capsulatum* could synthesise dextran from several MOS or partial acid hydrolysates of amylose, amylopectin and glycogen (Hehre, 1951). The enzyme converts chains of α(1→4)-linked glucosyl residues to new chains of α(1→6)-linked units. The extracellular enzyme isolated from *Gluconobacter oxydans* (ATCC 11894, NCIMB 4943) was initially named dextran dextrinase (DDase) but has been referred to as dextrin dextranase (1, 4-alpha-D-glucan: 1, 6-alpha-D-glucan 6-alpha-D-glucosyltransferase; EC 2.4.1.2) in more recent publications (Yamamoto *et al.*, 1992, 1993a, 1993b, 1993c, 1994a and 1994b; Suzuki *et al.*, 2000 and 2001). The reason for the departure from the more logical nomenclature is unclear since dextran dextrinase most aptly describes its mode of action (Naessens *et al*., 2005).

1.1 DDase mode of action

DDase is a transglucosidase which, as its main mode of action, converts maltodextrins to (oligo)dextran by catalysis of the transfer of an α(1→4)-linked non-reducing terminal glucosyl unit from an appropriate donor unit to a terminal glucosyl residue of

an acceptor molecule forming an α(1→6)-linkage (Yamamoto *et al.*, 1992). By repetitive action another non-reducing terminal α(1→4)-glucosyl residue is again transferred to the previous transfer product, forming an α(1→6)-linkage (Yamamoto *et al.,* 1993b; 1994a). By these consecutive transfers the dextran molecule is extended. Variable length substrate MOS are degraded step-wise to maltose which, in turn, is catalysed slowly to glucose and panose [α-D-glucose-(1→6)-α-D-glucose-(1→4)α-D-glucose] (Yamamoto *et al*., 1993b). The donor specificity and action mechanism was investigated by Yamamoto *et al.* 1994b. The researchers incubated several donor oligosaccharides and DDase with starch and salicin [2-(hydroxymethyl)phenyl-β-D-glucopyranoside] as shown in Table 1. Salicin contains a β-glucosidic residue which can not be hydrolysed by DDase action alone. Glucosyl residues were transferred to salicin and the results indicated that DDase could only transfer α(1→4)- or α(1→6)-linked glucosyl residues from donor to acceptor compounds.

Table 1. Donor and acceptor specificity of intracellular DDase from *G. oxydans* on selected oligosaccharides, methyl-α- and β-D-glucosides, starch and dextran where '+' denotes transglucosylation product formed and '–' indicates no transglucosylation product formed (Table reproduced from Yamamoto *et al.*, 1994b).

Compound	Transfer products	
	With salicin	With starch
Kojibiose	–	+
Sophorose	–	+
Nigerose	–	+
Laminaribose	–	+
Maltose	+	+
Cellobiose	–	+
Isomaltose	+	+
Gentiobiose	–	+
Trehalose	–	–
G-X [D-Glucosyl-α(1→4)-D-xylose]	–	+
Sucrose	–	+
Raffinose	–	–
Xylsucrose	–	+
Isoprimeverose [D-xylosyl-α-(1→6)-D-glucose]	–	+
Lactose	–	–
Melibiose	–	–
Methyl-α-D-glucoside	–	+
Methyl-β-D-glucoside	–	+
Salicin [2-(Hydroxymethyl)phenyl-β-D-glucopyranoside]	Not tested	+
Starch	+	Not tested
Dextran	+	Not tested

The introduction of α(1→4)-linkages as reported in dextran by Yamamoto *et al.,* (1992, 1993a) and Mountzouris *et al.* (1999) are derived from secondary modes of action for DDase as indicated in figure 1. Yamamoto *et al.* (1993b) elucidated two secondary modes of transglucosylation: (1) The transfer of an α(1→4)-linked glucosyl group to an acceptor with the subsequent formation of an α(1→4)-linkage and (2), the transfer of an α(1→6)-linked glucosyl group to an acceptor with the subsequent formation of an α(1→6)-linkage. These two secondary modes represent the disproportionation action on MOS and isoMOS, respectively. Yamamoto *et al.* (1993b) speculate that α(1→4)-linkages in dextran are formed as frequently as α(1→6)-linkages, but that the disproportionation reaction mentioned in (1) is reversed by the primary action of DDase. Dextran thus accumulates as α(1→6)-linked glucosyl residues as a result of the lower susceptibility of α(1→6)-linked glucosyl residues to cleavage. It is likely that DDase transglucosylates α(1→4)-linkages in dextran to MOS forming α(1→4)- and α(1→6)-linkages where dextran is the donor molecule and MOS the acceptor molecule (Jeanes *et al*., 1954; Yamamoto *et al.*, 1992). Dextran from *G. oxydans* consists of α(1→4)- and α(1→6)-linkages in a ratio of approximately 1:10 as shown by Mountzouris *et al*. (1999) while a 1:20 ratio is reported by Suzuki and co-workers (2001).

No dextran is produced from native starch while a yield of 21.4% has been reported with soluble starch as a substrate (Naessens *et al.*, 2005). The yield of dextran could be improved to 55 and 60% from starch and hydrolysed starch, respectively, in the presence of debranching enzymes such as iso-amylase and enzymes capable of endohydrolysis such as α-amylase and pullulanase (Yamamoto *et al.*, 1993a). Suzuki *et al.* (2001) report a yield of 73.9% using short chain amylose. These results suggest that DDase is able to act on non-reducing terminal residues of α(1→4)-linkages but not on α(1→4)-linkages near branching points in starch or soluble starch (Yamamoto *et al.*, 1993a).

1.2 Substrate specificity

DDase has been shown to act on a variety of substrates to yield dextran: MOS (G3 – G7), short chain amylose (G17.3) and, as mentioned above, soluble starch (Hehre, 1951; Yamamoto *et al.*, 1992; 1993a). Reduced MOS species consistently yield more

dextran than MOS (Yamamoto *et al.,* 1993b). Molecules with higher degrees of polymerisation (DP) display higher percentage yields of dextran when applied as a substrate as shown in Table 2 (Naessens *et al.,* 2005). Sims *et al.* (2001) suggests that the most effective maltodextrin substrate DP of three tested for dextran synthesis and resulting in the greatest yield percentage, is 28. A DP of 3 and 85 was also tested but resulted in lower yields of dextran. Glucose, fructose, sucrose, unhydrolysed amylose, amylopectin and maltose (G2) are shown to be unable to contribute glucosyl units to the extension of the dextran polymer (Hehre*,* 1951; Suzuki *et al.,* 2000; Naessens *et al*., 2005). Although maltose cannot contribute glucosyl units for dextran synthesis it is, nevertheless, hydrolysed and transglucosylated slowly by DDase to a free glucose unit and panose, a G3 molecule (Yamamoto *et al.,* 1992; 1993c; Suzuki *et al.*, 2000 and 2001). Maltose and its reduced form, maltitol (G2H), were unable to extend the dextran product, but several transfer products were formed (Yamamoto *et al.,* 1993b). The group speculated that G2 and G2H were reacted with dextran and the transfer products were elongated, resulting in dextran being degraded and subsequently reducing dextran yield. The hydrolysis and transglucosylation activity of DDase on maltose and maltitol seems to be interdependent and thus characteristic of the enzyme (Naessens *et al.,* 2005).

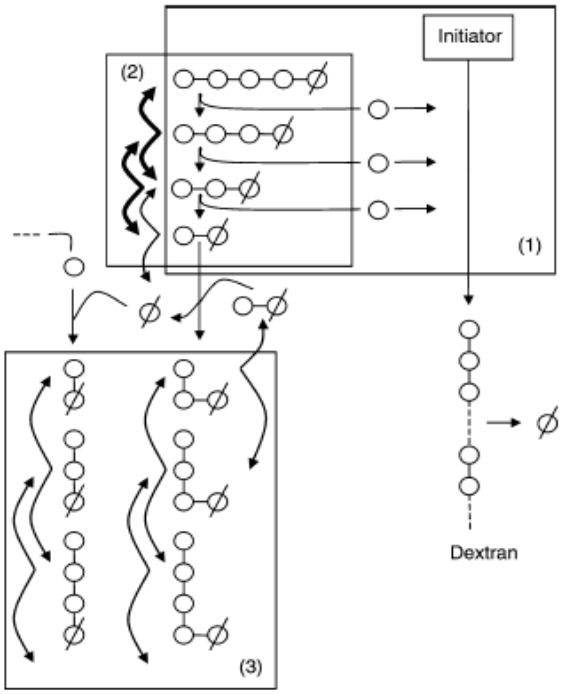

Figure 1. Summary of the three modes of action of DDase: (1) depicts the main mode of action where a α(1→4)-linked glucosyl unit is transferred to a α(1→6)-linked glucosyl residue of dextran. (2) Indicates α(1→4) to α(1→4) transfers and (3) shows α(1→6) to α(1→6) transfers. O indicates glucosyl residues and Ø glucose or reducing glucosyl residues. – depicts α(1→4)-glucosidic linkages; and I α(1→6) glucosidic linkages. Thick and thin lines indicate fast and slow reactions, respectively (Picture reproduced from Yamamoto *et al.,* 1992).

Table 2. Dextran yield when DDase is incubated with various substrates.

Substrate	Dextran extension	Dextran yield (%)
Soluble starch	Yes	21.4% (55%)[a]
Starch	No	0%
Short chain amylose	Yes	57.6%[b] (73.9%)[c]
Maltose (G2)	No	0%
Maltitol (G2H)	No	0%
Maltotriose (G3)	Yes	11%
Maltotetraose (G4)	Yes	13.4%
Maltotritol (G3H)	Yes	22.4%
Maltotetraitol (G4H)	Yes	36.8%
Maltopentaose (G5)	Yes	25%
Maltohexaose (G6)	Yes	30.2%
Maltoheptaose (G7)	Yes	N/A
Glucose	No	0%
Dextrin	Yes	N/A

a is yield when incubated with iso- or α-amylase and pullulanase. **b** the average DP of the short chain amylose is 17.3. **c** as recorded by Suzuki *et al*., 2001, no DP recorded.

An initiator or primer molecule for dextran synthesis has been proposed by Naessens *et al.* (2005). The primer which acts as the glucosyl acceptor molecule for the first transglucosylation reaction forming an α(1→6)-linkage to initiate further dextran synthesis has not been confirmed. If, for example, maltotetraose (G4) is used as a substrate, glucose-α(1→6)-maltotetraose – or other MOS – that may be produced by the first disproportionation reaction might be transglucosylated to a glucose-α(1→6)-maltooligosaccharide and elongation of the dextran molecule may then proceed (Naessens *et al.*, 2005).

Extracellular DDase activity has been tested for a range of MOS (Suzuki *et al.*, 2000). The K_m values decrease with increasing lengths of MOS. Maltotriose has a K_m of 10.2mM while short chain amylose with an average chain length of 17.3 glucosyl units has a K_m of 0.12mM. The K_m for MOS is assumed to follow a non-linear trend of decreasing value with an increasing DP as shown in figure 2. Sims *et al.* (2001), as mentioned previously, demonstrated that of the three average degrees of polymerisation tested dextran yield peaked at a DP value of 28 and then decreased with a DP of 85 on day 5, when total dextran yield was at a maximum. The K_m values for these MOS were not calculated and the full range of DP was not explored, thus optimal DP for dextran yield with DDase can not be estimated accurately.

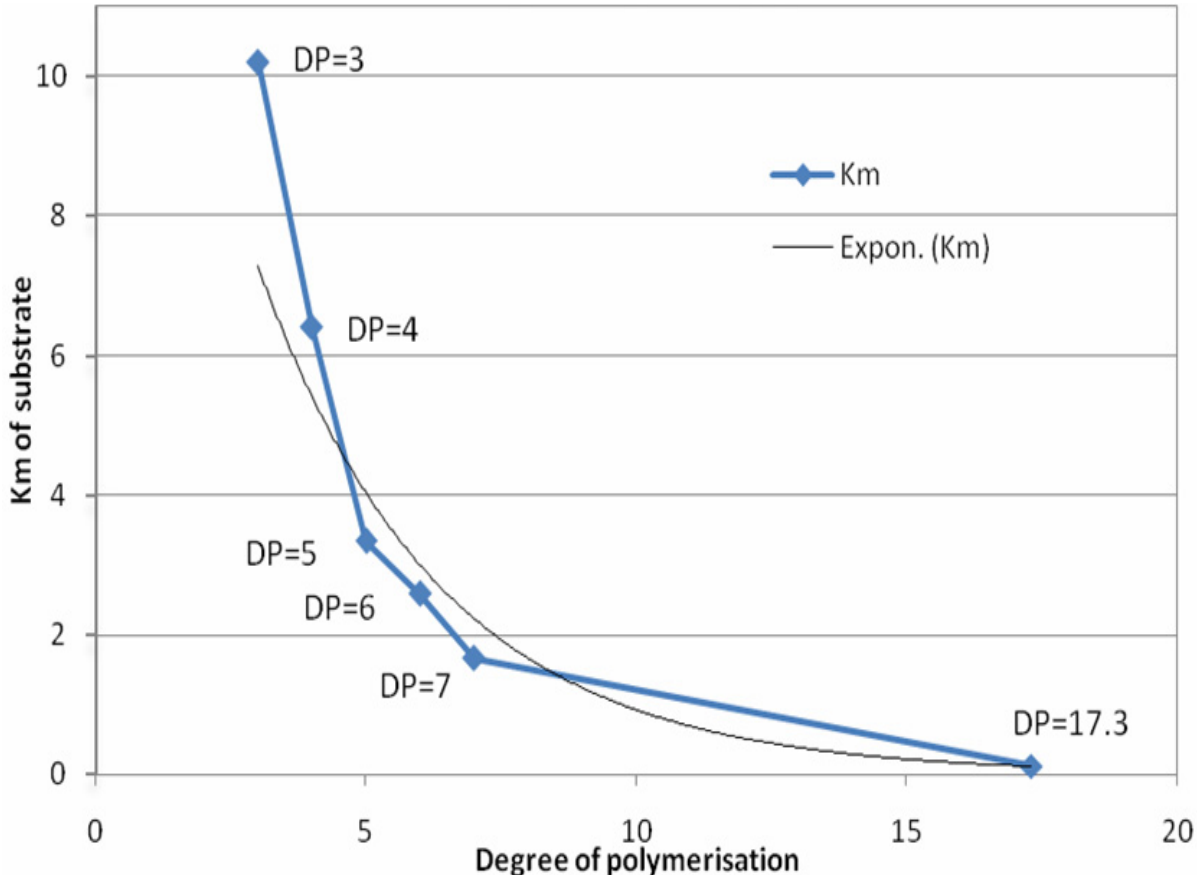

Figure 2. Graph of K_m values for several MOS and short chain amylose with increasing DP values. DP values of 3, 4, 5, 6, 7 and 17.3 are maltotriose, -tetraose, -pentaose, -hexaose, -heptaose and short chain amylose, respectively. Also shown is a hyperbolic trend line indicating estimated K_m values for intermediate DP substrates.

1.3 DDase isozymes

DDase from *G. oxydans* is produced in two forms depending on fermentation conditions: intracellular ($DDase_{int}$) and extracellular ($DDase_{ext}$) (Yamamoto *et al.*, 1992). It has been shown that intracellular DDase enzyme is produced at the onset of the log phase. Interestingly, when cultures of *G. oxydans* were incubated with iso- or MOS $DDase_{int}$ yields decreased rapidly upon addition and the bulk of DDase enzyme activity was detected extracellularly (Naessens *et al.*, 2005). The rapid response to increased levels of added MOS led Naessens and co-workers (2002, 2003, and 2005) to believe that the intracellular enzyme is secreted into the extracellular environment. The apparent secretion is not prevented by the addition of energy uncoupling agents, suggesting a passive secretion mechanism (Naessens *et al.*, 2005). After incubation in a low molarity buffer such as 10mM Na-acetate, $DDase_{int}$ is liberated into the buffer solution (Naessens, 2003). Upon secretion of the enzyme in a low molarity buffer and

in the absence of substrates and dextran the enzyme is thought to adsorb to the *G. oxydans* cells (Naessens *et al.*, 2005). This adhesion is apparently not due to ionic interactions between the cells and the enzymes (Naessens *et al.*, 2005). The relationship between $DDase_{int}$ and $DDase_{ext}$, however, remains unclear since the loss of $DDase_{int}$ activity, after the cell culture is supplemented with MOS, does not result in a reciprocal increase in $DDase_{ext}$ activity as would be expected from a secretory system.

It is possible that the production of an extracellular version of DDase is up-regulated and $DDase_{int}$ down-regulated on the addition of MOS (Naessens *et al.*, 2002; 2005). The apparent secretion and adsorption of $DDase_{int}$ to the cell occurred in a low molarity cell suspension and in the absence of substrate and dextran; however, discrepancies in enzyme activities exists even when the cells were cultured with MOS and dextran.

Evidence suggesting that the DDase isoforms are representative of two different genes rests primarily on molecular weight evidence from SDS-PAGE. $DDase_{int}$ has a molecular weight of approximately 300 kDa (Yamamoto *et al.*, 1992) while $DDase_{ext}$ has a weight of 152 kDa (Suzuki *et al.*, 2001). Furthermore, $DDase_{ext}$ lacks the disproportionation activity of $DDase_{int}$, lending some weight to a two gene theory (Naessens *et al.*, 2005).

To settle the debate over whether $DDase_{ext}$ is simply the secreted form of $DDase_{int}$ that undergoes some degree of processing or whether they are encoded for by different genes requires amino acid sequence information and the identification of the encoding gene(s).

1.4 Temperature and pH optima

The DDase isoforms not only share a similar mode of action, but also share similar temperature as well as pH optima (Suzuki *et al.,* 2001). Intracellular DDase activity was noted by Yamamoto *et al.* (1992) as having a temperature optimum of 37–45°C and the protein retains stability in temperatures below 45°C. The intracellular enzyme is stable at a pH range of 3.5–5.2 with an optimum of 4.0–4.2 at 30°C for 30 minutes (Yamamoto *et al.*, 1992). Extracellular DDase activity exhibits a temperature and pH optimum of 38°C and 5.2, respectively (Naessens *et al.*, 2005). The enzyme retains its original activity up to 45°C, and the protein remains stable in the pH range of 4.1–5.2 at

4°C for 24 hours. The enzyme appears to be hydrophobic since it was shown to be stable during an *n*-butanol extraction step (Yamamoto *et al.,* 1992 and 1993c).

1.5 Inhibitors and activators

Intracellular DDase activity is found to be strongly inhibited by Fe^{3+} but activity is only mildly suppressed by 0.5mM concentrations of Co^{2+}, Zn^{2+} and Mg^{2+}, 1.0mM EDTA, and 0.2mM ρCMB (ρ-Chloromercuribenzoic acid; Yamamoto *et al.*, 1992). The enzyme is slightly activated by 0.5mM Mn^{2+} (Yamamoto *et al.*, 1992). Extracellular DDase activity is found to be completely inactivated by 1mM concentrations of Hg^{2+}, $KMnO_4$ and Pb^{2+} (Naessens, 2003). $DDase_{ext}$ is found to be partly inhibited by 1mM concentrations of Cd^{2+}, Cu^{2+} and Zn^{2+}. The effect of Fe^{3+} on $DDase_{ext}$ was not investigated. Maltose is a strong inhibitor of dextran synthesis, since transglucosylation results in the production of glucose and panose where dextran glucosyl residues are transglucosylated to maltose to synthesize panose (Suzuki *et al.*, 2000).

1.6 Glycosyltransferases from the *G. oxydans* genome

Several unclassified glycosyltransferases from the G. oxydans genome have been sequenced and recorded (http://gib.genes.nig.ac.jp/single/index.php?spid=Goxy_621H, http://cmr.tigr.org/tigr-scripts/CMR/shared/GeneList.cgi?sub_role=90&sub_org_val=ntgo01). Ten of the sixteen glycosyltransferases are noted simply as putative glycosyltransferases while the unclassified remaining six are recorded as glycosyltransferases. All of the protein sequences display one or more conserved domain sequence (CDS) as shown in Table 3. These are pfam00534 and pfam00535 which are glycosyltransferase group 1 and glycosyltransferase family 2 CDS, respectively. The rfaG CDS is a glucosyltransferase I. The PRK10422 CDS is involved in lipopolysaccharide biosynthesis and belongs to glycosyltransferase family 9. COG1216 is a predicted glycosyltransferase CDS.

Table 3. Glycosyltransferases and putative glycosytransferases from internet genome sources. Included is the gene name, putative function, molecular weight and conserved domain sequence.

Glycosyltransferases and putative glycosyltransferases				
Gene name	EC number	Putative function	Calculated MW (kDa)	CDS
GOX0593	2.4.1.-	Putative glycosyltransferase	83.8	1, 2
GOX0595	2.4.1.-	Putative glycosyltransferase	124.33	1, 3
GOX0710	2.4.1.-	Putative glycosyltransferase	40.48	1
GOX0712	2.4.1.-	Glycosyltransferase	97.9	1, 2
GOX0897	2.4.1.-	Putative glycosyltransferase	46.05	4
GOX0939	2.4.1.-	Glycosyltransferase	71.97	1, 3
GOX0999	2.4.1.-	Putative glycosyltransferase	39.7	4
GOX1049	2.4.1.-	Glycosyltransferase	35.5	2
GOX1123	2.4.1.-	Glycosyltransferase	102.65	1, 2, 3
GOX1125	2.4.1.-	Glycosyltransferase	99.75	1, 3
GOX1482	2.4.1.-	Glycosyltransferase	41.3	1
GOX1489	2.4.1.-	Putative glycosyltransferase	80.58	5
GOX1490	2.4.1.-	Putative glycosyltransferase	61.43	2
GOX2128	2.4.1.-	Putative glycosyltransferase	34.71	2
GOX2238	2.4.1.-	Putative glycosyltransferase	114.52	1, 2
GOX2254	2.4.1.-	Putative glycosyltransferase	41.77	2, 5

The CDSs are pfam00534 (1), pfam00535 (2), rfaG (3), PRK10422 (4) and COG1216 (5).

1.7 Applications of *G. oxydans* dextran

The relatively high proportion of α(1→6)-linkages in dextran makes it an ideal non-nutritive bulking and sweetening agent in foods. Dextran is less prone to hydrolysis by salivary and intestinal enzymes and microflora while the digestion of maltodextrins, often used in foods, is more or less complete (Mountzouris *et al.,* 1999; Yamamoto *et al.*, 1993c). *G. oxydans* dextran may also be used as a source of dietary fibre, a fat substitute and as a cryostabiliser (Naessens *et al.,* 2005). Dextran has many medical applications. It can be used as a plasma volume expander to limit anaphylactic shock from excessive blood loss and it can be used as a medium to administer large doses of iron into anaemic patients as a ferric dextran. Dextran is also used in size-exclusion chromatography matrices and as an immobilisation medium for biosensors. Dextran can also be used to coat metal nanoparticles and thus protect them from oxidation and enhance biocompatibility.

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Chapter 2

Section 1

2.1 Introduction

The spoilage of beer has long been associated with acetic acid bacteria from the *Gluconobacter* (renamed from *Acetobacter*) genus (Baker *et al*., 1912 and Shimwell, 1947) The spoilage, termed 'ropiness', is caused by the production of capsular material by bacteria from dextrins naturally found in beer. The capsular material has been shown to consist largely of maltooligosaccharides (MOS) and dextran which is immunologically similar to dextran produced by *Leuconostoc mesenteroides* by action of dextransucrase (DSase; Shimwell, 1947; Hehre and Hamilton, 1949). It was later shown that a partially purified extracellular protein from a *Gluconobacter* species could synthesise dextran from short chains of MOS and other glucosyl compounds. The enzyme was shown to be able to convert chains of α(1→4)-linked glucosyl residues to new chains comprising of α(1→6)-linked units. The extracellular enzyme isolated from *G. oxydans* (ATCC 11894) was initially named dextran dextrinase (DDase) but has been referred to as dextrin dextranase in some more recent publications (Yamamoto *et al.*, 1992; Yamamoto *et al.,* 1993a, b and c; Yamamoto *et al.,* 1994a and b; Suzuki *et al.*, 2000; Suzuki *et al.*, 2001). The reason for the departure from the more logical nomenclature is unclear (Naessens *et al*., 2005).

DDase, as a transglucosidase, converts maltodextrins to oligodextrins by the preferential transfer of an α(1→4)-linked non-reducing terminal glucosyl unit from a donor molecule to a terminal glucosyl residue of an acceptor molecule, forming an α(1→6)-linkage (Yamamoto *et al.*, 1992). The dextran molecule is then synthesised by the repetition of the transglucosylation reaction. Two secondary modes of transglucosylation have been elucidated: (1) The transfer of an α(1→4)-linked glucosyl group to an acceptor with the subsequent formation of an α(1→4)-linkage and (2), the transfer of an α(1→6)-linked glucosyl group to an acceptor with the subsequent formation of an α(1→6)-linkage (Yamamoto *et al.*, 1993b). These secondary modes represent the disproportionation action on MOS and isoMOS (MOS containing

α(1→6)-linkages), respectively, of DDase. It has been speculated that α(1→4)-linkages in dextran are formed as frequently as α(1→6)-linkages by DDase, but that the disproportionation reaction, where α(1→6)-linkages are formed, is reversed by the primary action of DDase (Yamamoto *et al.*, 1993b). Dextran therefore accumulates α(1→6)-linked glucosyl residues as a result of the lower susceptibility of α(1→6)-linked glucosyl residues to cleavage by the secondary mode of DDase. DDase has been shown to be able to act on a variety of substrates to yield dextran (Hehre, 1951; Yamamoto *et al.*, 1992; 1993a). MOS (G3 – G7; where G represents the number of glucosyl units), short chain amylose (G17.3) and soluble starch all result in the extension of the dextran molecule (Hehre, 1951; Yamamoto *et al.*, 1992; 1993a). Reduced MOS species (sugar alcohols) yield more dextran than MOS while molecules with higher degrees of polymerisation (DP) display higher percentage yields of dextran when used as substrate (Yamamoto *et al.,* 1993b). Simple sugars such as glucose and fructose as well as disaccharides such as sucrose and maltose and glucosyl polymers such as unhydrolysed amylose and amylopectin are not able to contribute to dextran polymer extension (Hehre*,* 1951; Suzuki *et al.,* 2000; Naessens *et al*., 2005). Maltose, although unable to be utilised by DDase for dextran production, is hydrolysed and transglucosylated to glucose and panose. Panose is speculated to serve as a primer molecule if MOSs were to be introduced to the bacterial environment, thus seeding the culture for dextran synthesis (Naessens *et al*., 2005).

DDase was shown to be produced in two forms: an extracellular ($DDase_{ext}$) and an intracellular ($DDase_{int}$) version (Suzuki *et al.*, 2001; Yamamoto *et al.*, 1992). $DDase_{ext}$ activity has been tested for several MOSs (Suzuki *et al.*, 2000). K_m values were found to decrease with increasing chain lengths of MOS. For example the K_m of the enzyme for maltotriose of 10.2mM while short chain amylose with an average chain length of 17.3 glucosyl units was calculated to have a K_m of 0.12mM. The K_m for MOS is assumed to follow a non-linear trend of decreasing value with an increasing degree of polymerisation (DP; Suzuki *et al.*, 2000; Sims *et al.*, 2001).

$DDase_{int}$ is produced at the onset of the log phase of growth but yields of $DDase_{int}$ decrease rapidly upon addition of isoMOS or MOS to cultures of *G. oxydans*. The bulk of DDase enzyme activity is then detected extracellularly (Naessens *et al.*, 2005). It is

assumed that the enzyme is excreted into the extracellular environment after some unknown modification(s), however, the relationship between $DDase_{int}$ and $DDase_{ext}$ remains unclear since the loss of $DDase_{int}$ activity after the cell culture is supplemented with MOS does not result in a similar increase in $DDase_{ext}$ activity as would be expected by its secretion (Naessens *et al.*, 2005). This secretion is also not prevented by energy uncoupling agents, suggesting a possible passive mechanism, and after incubation in a low molarity buffer $DDase_{int}$ was found to be liberated into the buffer solution (Naessens, 2003). If the enzyme is secreted then it is thought to adsorb to the *G. oxydans* cells when there is absence of substrates in the medium (Naessens *et al.*, 2005).

Evidence of the DDase isoforms being representative of two different genes rests primarily on their molecular weights estimated from SDS-PAGE, where the external form is approximately 150 kDa and the internal form 300 kDa (Suzuki *et al.*, 2001). Furthermore, $DDase_{ext}$ lacks the disproportionation activity found in $DDase_{int}$, lending some weight to a two gene theory (Naessens *et al.*, 2005).

The complete genome of *Gluconobacter oxydans* (*G. oxydans* ATCC 621H) has recently been sequenced and published (Prust *et al.* 2005). It is approximately 2.7 Mbp excluding four plasmids of 26.6, 14.5, 13.2 and 2.7 Kbp and one megaplasmid of 163 Kbp. Several excellent *G. oxydans* genome websites exist such as the Genome Information Broker – Microbial Genomes (GIB-M) and Comprehensive Microbial Resource (CMR) (http://gib.genes.nig.ac.jp/single/index.php?spid=Goxy_621H, http://cmr.tigr.org/tigrscripts/CMR/shared/GeneList.cgi?sub_role=90&sub_org_val=ntgo01). According to CMR, some 17.75% of genes on the *G. oxydans* genome are noted as of having hypothetical function, 1.96% of expressed genes are not assigned a role category and 1.15% are conserved hypothetical genes. The aim of this project is to try and identify which of the genes in the *G. oxydans* genome codes for the DDase.

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Chapter 2

Section 2

2.2.1 Materials

All chemicals were obtained from Sigma-Aldrich (South Africa) unless otherwise indicated.

All DNA modifying enzymes were from Promega (South Africa) unless otherwise indicated.

Primers were purchased from Integrated DNA Technologies (IDT, Whitehead Scientific, South Africa).

Gluconobacter oxydans strain ATCC 19357 was a gift from Christie Malherbe (Department of Biochemistry, Stellenbosch University, Stellenbosch) and ATCC 621H was obtained from DSMZ, Germany.

The *E. coli pgm* mutant strain CGSC# 5527, designation PGM1, was obtained from Yale *E. coli* genetic stock centre.

2.2.2 Methods

2.2.2.1 Extraction of proteins from cell-free culture.

A 5mL *G. oxydans* (ATCC 19357 or ATCC 621H) pre-culture was added to 500mL growth media (0.5% (w/v) yeast extract, 0.5% (w/v) bacteriological peptone, 5.5% (w/v) glucose, and 0.05% (w/v) soluble dextrin) and incubated for 24 hours at 28°C. The cell culture was centrifuged at 2 000*g* for 20 minutes at 4°C to pellet the cells (Yamamoto *et al.*, 1992).

The cell-free culture supernatant was centrifuged at 20 000*g* for 30 minutes at 4°C. The pellet containing the enzyme activity was resuspended in chilled 50mM Na-acetate

buffer; pH 4.5 (50mM sodium acetate - anhydrous, pH with glacial acetic acid). This was filtered through a 0.45µm filter (Lasec SA, South Africa) after which glycerol was added to a final concentration of 10% (v/v).

2.2.2.2 SDS-PAGE and activity gel analysis

Proteins were separated on 4% resolving and 8% separating PAGE gels unless indicated otherwise. SDS-PAGE gels were run according to Sambrook and Russell, 2001. Activity gels were cast and run similarly to SDS-PAGE gels; however, SDS was not included in the separating and resolving gels or running buffer. Loading buffer (250mM Tris; pH 6.8, 40% (v/v) glycerol, 0.01% (w/v) Bromophenol Blue) for activity gels did not contain any SDS, DTT or β-mercaptoethanol and samples were not heat-denatured prior to loading. All PAGE gels were run at 150V for the duration of the separation on a Hoefer® miniVE system. Activity gels were electrophoresed at 4˚C and SDS-PAGE at room temperature.

2.2.2.3 Agarose gel DNA electrophoresis

All gels contained 1% (w/v) agarose (D-1 Low EEO; Conda, Spain), unless indicated otherwise and 0.01% (v/v) ethidium bromide in TBE buffer. DNA samples were separated by electrophoresis. All samples were loaded using 5% (v/v) standard DNA loading dye (30% (v/v) glycerol, 0.25% (w/v) bromophenol blue in ddH_2O).

2.2.2.4 Cloning of putative DDase gene into pGEX-4T-1

2.2.2.4.1 PCR of putative gene

A putative DDase sequence (GOX0496) from *Gluconobacter oxydans* (ATCC 621H) was amplified from a sequence identified through NCBI using PCR with the following primers: DDase pGEX Fwd 5’ GAATTCATATGGCGTCAAAGCTTCTC’3 and DDase Rev 5’ TCAGCCGGCTTTCTCCAG 3’. Elongase taq polymerase (Invitrogen) was used to amplify the 5274 base-pair sequence using a GeneAmp PCR System 9700 (Applied Biosystems). Amplification conditions were: 94°C for 2 minutes; 40 x (94°C

for 30 seconds, 46°C for 2 minutes, 68°C for 30 seconds); 68°C for 5 minutes. The amplification product was separated on a 1% (w/v) agarose gel, excised and purified with the QIAquick Gel Extraction Kit (Qiagen) as per manufacturer's instructions.

2.2.2.4.2 Ligation of PCR product into pGEM-T Easy

The amplification product was ligated into pGEM-T Easy (Promega) using T4 DNA ligase (Promega). Insert-containing plasmids were identified through blue/white selection on Luria broth (LB) agar media containing 40µg/mL X-gal, 0.5mM IPTG and 100µg/mL ampicillin. White colonies were used to inoculate 5mL of Luria broth containing 100µg/mL ampicillin and grown overnight at 37˚C with shaking.

2.2.2.4.3 Plasmid purification

Plasmids were extracted and purified using the plasmid boiling miniprep method (Holmes and Quigley, 1981) after which they were visualised on an agarose gel. 5mL of culture was pelleted by centrifugation in a 1.5mL microcentrifuge tube at 16 000*g* for 5 minutes, the supernatant was discarded and the pellet resuspended in 300µL of STET buffer (8% (w/v) sucrose, 5% (v/v) Triton X-100, 50mM EDTA, 50mM Tris; pH 8.0 and 15 000 units of lysozyme). The samples were immediately boiled for 90 seconds and centrifuged at 16 000*g* at room temperature for 15 minutes. The supernatant was transferred to a new microcentrifuge tube to which 1 volume of isopropanol was added and centrifuged again at 16 000*g* for 10 minutes. The supernatant was removed and the pellet resuspended in 50µL ddH_2O.

2.2.2.4.4 Restriction analysis and ligations

Plasmids were digested with restriction enzymes (all from Promega) according to the manufacturer's instructions. Isolated fragments were ligated into vectors using T4 DNA ligase (Promega) according to the manufacturer's instructions.

2.2.2.4.5 Production and partial purification of GST-fusion proteins

The insert-containing pGEX-4T-1 plasmid was transformed into heat-shock competent *E. coli* BL-21 cells and selected for on LB-ampicillin plates (100µg/mL ampicillin).

The GST-fusion protein was generated using a heat shock treatment. A 2mL pre-culture was used to inoculate 200mL of 'Terrific broth'-ampicillin-chloramphenicol media (1.2% (w/v) bacto-tryptone, 2.4% (w/v) bacto-yeast extract, 0.4% (v/v) glycerol, 17mM KH_2PO_4 monobasic, 7.2mM $K_2HPO_4 \cdot 3H_2O$ and 100µg/mL ampicillin and chloramphenicol) and grown to an OD_{600} of 0.1 at 37˚C with shaking. The media was then grown to an OD_{600} of 0.5 at 42˚C with shaking after which the culture was induced by addition of IPTG to a final concentration of 0.3mM for 5 hours with shaking at 16˚C. The culture was pelleted at 1000*g* for 10 minutes, resuspended in 0.05 volumes of PBS buffer (10mM K-phosphate buffer; pH 7.4, 150mM NaCl) and the cells lysed by sonication.

The GST-fusion protein was purified using glutathione-agarose (Sigma-Aldrich) column purification. The fusion protein solution was incubated overnight at 4˚C with 300µL hydrated glutathione-agarose with gentle shaking and partially purified as per manufacturer's instructions. The flow-through, two wash fractions and two reduced glutathione eluted fractions were analysed by SDS-PAGE and immunoblotting.

2.2.2.5 Immunoblots

Protein was denatured by addition of 0.1 volume loading buffer (250mM Tris; pH 6.8, 8M urea, 40% (v/v) glycerol, 0.01% (w/v) Bromophenol Blue and 2% (v/v) β-mercaptoethanol) at 95˚C for 5 minutes before separation by SDS-PAGE. Tris-glycine electrophoresis buffer (25mM Tris, 250mM glycine; pH 8.3, 0.1% (w/v) SDS) was used for all SDS-PAGE gels. Gels were transferred to Immobilon-P (Millipore) PVDF membranes in transfer buffer (48mM Tris, 39mM glycine, 20% (v/v) methanol and 0.0375% (w/v) SDS) using a Trans-Blot SD semi-dry electrophoretic transfer cell (Bio-Rad).

Membranes were blocked in 3% (w/v) BSA (Bovine Albumin (Fraction V) (Roche)) in TBST-buffer (20mM Tris; pH 7.6, 137mM NaCl, 0.1% (v/v) Tween-20) for 2 hours. The primary antibody (1:3000, polyclonal mouse anti-GST, gift from Dr A. de Beer,

Department of Wine Biotechnology, University of Stellenbosch, Stellenbosch) was added to the above buffer, incubated overnight at 4˚C with gentle shaking and rinsed in TBST-buffer. The secondary antibody (1:30 000, alkaline phosphatase conjugated goat anti-mouse-IgG, Sigma-Aldrich) in TBST-buffer containing 3% (w/v) low fat milk powder was then added and incubated for 1 hour. The membrane was rinsed with TBST-buffer, washed twice in TBST-buffer containing 0.05% (w/v) SDS and twice in TBST-buffer. Blots were developed using NBT/BCIP (Roche).

2.2.2.6 Functional analysis of genes in the *E. coli pgm* mutant

Plasmids were transformed into the *pgm* mutant of *E. coli* and transformants were grown on LB plates supplemented with 100mM maltose and 50µg/mL ampicillin. The plates were stained with iodine (Sigma) vapour.

2.2.2.7 Kinetic parameters of the enzyme using maltotriose as a substrate

A 50mM stock of maltotriose in 10mM Na-acetate, pH 4.5, was used to create a substrate concentration series in triplicate with final concentrations of 1, 5, 10, 15, 20 and 25mM. After addition of 7µg of the partially purified DDase protein (50µl of 0.14µg/µL protein stock (Section 2.2.2.1)) the final reaction volume was 400µL. Protein concentration determined by Bradford (1976; Section 2.2.2.23). The solution was incubated at 37˚C for 1 hour. After the incubation period the assay was stopped by the addition of 3 volumes of ethanol and allowed to precipitate overnight at room temperature. The dextran product was then washed once with 80% (v/v) ethanol and centrifuged at 16 000*g* at room temperature. The pellet was resuspended in 400µL water. A sample of dextran solution was hydrolyzed to glucose (Section 2.2.2.10) and the amount of glucose present was determined enzymatically using hexokinase and glucose 6-phosphate dehydrogenase (Section 2.2.2.11). The enzyme activity was measured as µg dextran formed/ µg protein/ minute and K_m was determined.

The activity was plotted against the increasing substrate concentrations on SigmaPlot (Version 7.0, Systat Software Inc.) (two-parameter, rectangular hyperbola) to obtain a hyperbolic curve. From this the V_{max} and K_m (0.5 V_{max}) for maltotriose were determined.

2.2.2.8 Assay using ρ-Nitrophenyl-α-D-glucopyranoside (NPG) as a substrate

A stock solution of NPG was diluted into wells of a 96 well mictrotitre plate to a final substrate concentration of 40mM NPG with 10mM Na-acetate; pH 4.5. Standardised masses of sample protein were added and incubated at 25°C. Readings were taken every minute for 1 hour and the linear section of the graph used for activity calculations. The absorbance of ρ-nitrophenyl was read at 400nm and the molar extinction coefficient of NPG at OD_{400} is 18 380 M^{-1} cm^{-1}. The activity was defined as: $1U_{NPG}$= the amount of enzyme liberating 1µmole of nitrophenyl from NPG per minute under standard assay conditions (Naessens *et al.*, 2002)

2.2.2.9 Dextran production

100µL of a buffer containing either maltotriose or MOS (10mM Na-acetate, pH 4.5, 100mM maltotriose or 5% (w/v) MOS (Merck)) was incubated with 7µg (50µL of 0.14µg/µL protein stock, Section 2.2.2.1) DDase. 10µL of purified 0.14µg/µL DDase protein was added every two hours for 5 hours and incubated with gentle shaking at room temperature overnight. The sample was heated to 90°C for 10 minutes to inactivate the enzyme and 3 volumes of ethanol were added. After precipitation at room temperature overnight the sample was centrifuged at 16 000*g* in a desktop centrifuge for 10 minutes at room temperature. The pellet was washed using 80% (v/v) ethanol and partially dried after which the pellet was resuspended in water.

2.2.2.10 Glucose content of hydrolysed dextran samples

Dextran sample obtained by reacting DDase with maltotriose or MOS (Section 2.2.2.9) were hydrolysed to measure glucose content. 25uL of sample was hydrolysed by addition of 50µL 0.7M HCl at 95°C for 4 hours with frequent mixing. The reaction was then neutralized by addition of 50µL 0.7M KOH. The samples were then assayed for free glucose as described later (Section 2.2.2.11).

2.2.2.11 Assay for free glucose

Glucose was determined enzymatically by measuring at 340nm in a microtitre plate reader (PowerWave$_x$, Bio-Tek Instruments). 200µL of assay buffer (150mM Tris, pH 8.1; 5mM $MgCl_2$, 0.4mM NAD, 1.6mM ATP), 45µL water and 5µL sample was placed in a well. The plate was read at A_{340} before the addition of 0.5U hexokinase/ glucose-6-phosphate dehydrogenase mixture (Roche). The mixture was allowed to incubate at room temperature for 30 minutes after which the absorbance was read again. The glucose content was calculated from the difference in the absorbance before and after incubation with the enzymes (Bergmeyer and Bernt, 1974).

2.2.2.12 Activity with GST-fusion protein

Approximately 50µL of a 0.1µg/µL protein stock (Section 2.2.2.4.5) of the fusion protein was incubated with a maltose or MOS solution (10mM Na-acetate; pH 4.5, 100mM maltose or 5% (w/v) MOS (Merck)) overnight. Samples incubated with maltose were analysed enzymatically for free glucose, as described previously (Section 2.2.2.11), while the MOS containing buffer/ GST fusion protein was analysed for dextran as described previously (Section 2.2.2.10).

2.2.2.13 Digestion of dextran with dextranase

2.2.2.13.1 Digestion of dextran with dextranase in vitro

Dextranase from *Penicillium* sp. (Sigma-Aldrich) was used to hydrolyse dextran obtained from reacting DDase with maltotriose or MOS. 25µL of the resuspended sample (Section 2.2.2.1) was added to 265µL of 50mM Na-acetate buffer; pH 6.0, and incubated with 150 units of dextranase for 2 hours at 37°C with occasional gentle mixing. The sample was then heated to 100°C for 10 minutes to inactivate the enzyme and the products analysed by HPLC and TLC. As a negative control protein samples were incubated without substrate.

2.2.2.13.2 Digestion of dextran with dextranase in gel

Dextran synthesised in an activity gel by the reaction of MOS with DDase (Section 2.2.2.9) was digested with 150 units of dextranase in 50mM sodium acetate buffer; pH6.0, overnight.

2.2.2.14 Digestion of isomaltose with α-Glucosidase (Type I)

α-Glucosidase from *Saccharomyces cerevisiae* (Type I, Sigma-Aldrich) was used to specifically hydrolyse isomaltose (formed in the hydrolysis of dextran by dextranase) to glucose. 90µL of the sample previously digested (Section 2.2.2.13) with dextranase was incubated with 10 units of α-glucosidase in 50mM Na-acetate buffer; pH 6.8, at 37°C for 2 hours in a final volume of 150µL. The sample was then heated to 100°C for 10 minutes to inactivate the enzyme and analysed by HPLC and TLC.

2.2.2.15 Analysis by Thin Layer Chromatography (TLC)

10µL of sample (Section 2.2.2.13 and 2.2.2.14) was applied to a TLC plate (Silica Gel 60 Adamant, Fluka) and dried. Standards containing a total of 3.6µg glucose and 1.9µg maltose were run. TLC plates were run with a soluble phase of ethyl acetate:glacial acetic acid:H_2O (10:5:6) and dried. The plate was developed at 100°C for 30 minutes after application of ρ-anisaldehyde reagent (93% (v/v) EtOH, 3.5% (v/v) H_2SO_4, 1% (v/v) acetic acid, 2.5% (v/v) ρ-anisaldehyde).

2.2.2.16 Schiff staining

Two 6%, non-denaturing PAGE gels with 0.4 and 1.7µg of protein per well was electrophoresed at 4°C. One gel containing 10mg/mL MOS and 0.001% (w/v) dextran primer was used to detect in-gel polymer production while the control gel was cast without MOS substrate or primer. After electrophoresis both gels were washed in fixing solution (40% (v/v) methanol, 7% (v/v) acetic acid) for 30 minutes. The gels were then washed twice in 10mM Na-acetate; pH 4.5, for 30 minutes and incubated overnight at 37°C in 10mM Na-acetate/ MOS buffer; pH 4.5, for the activity gel and 10mM Na-

acetate buffer; pH 4.5, for the control, respectively. After incubation the gels were washed once in fixing solution for 30 minutes before being placed in a solution of 1% (w/v) periodic acid and 3% (v/v) acetic acid and washed for 60 minutes. The gels were then removed from the solution and washed 10 times in water for five minutes. Following this they were then immersed in Schiff's reagent (Sigma-Aldrich) in the dark. The gels were removed after approximately 10 minutes when staining bands were observed and the reaction was stopped by washing in excess water.

2.2.2.17 Genomic DNA extraction

G. oxydans (ATCC 19357) was cultured in 500mL of broth (5.5% (w/v) glucose, 0.5% (w/v) yeast extract, 0.5% (w/v) bacteriological peptone) for 20 hours at 28°C. The cells were centrifuged at 2000*g* for 15 minutes, washed with 50mL of buffer solution 1 (10mM Tris, 1mM EDTA) and resuspended in 25mL of buffer solution 2 (10mM Tris, 20mM EDTA).

The cell suspension was treated with 1mL of lysozyme solution (10mg/mL) at 37°C for 30 minutes followed by treatment with 400 units of proteinase K at 37°C for 30 minutes and 5mL of 5% (w/v) SDS at 37°C for 1 hour. The DNA was extracted twice with 30mL of phenol: chloroform: isoamyl alcohol (25:24:1) by gentle shaking at 4°C for 10 minutes and centrifuged at 10 000*g* for 20 minutes. The supernatant was then further extracted with 30mL of chloroform: isoamyl alcohol (24:1).

25mL of the supernatant obtained by centrifugation was mixed with 2.5mL of 3M sodium acetate (pH 7.0) to which 55mL of ice-cold absolute ethanol was added. The DNA was obtained by winding using a glass rod and was then treated with 0.2mg RNaseA (Sigma-Aldrich) at 37°C for 30 minutes. The phenol chloroform extractions were repeated to obtain pure chromosomal DNA while the last extraction was replaced by a chloroform: isoamyl alcohol (24:1) wash to remove the remaining phenol. The DNA concentration was determined spectrophotometrically.

2.2.2.18 Tryptic digest

The tryptic digest was performed following the method published by Shevchenko *et al.* (1996). Protein bands stained with Coomassie colloidal blue (0.1% (w/v) Coomassie

brilliant blue R-250, 40% (v/v) methanol, 10% (v/v) acetic acid) were excised from the gel and cut into smaller fragments. The gel fragments were destained in 500µL destain (40% (v/v) methanol, 10% (v/v) acetic acid) for several hours with regular changing of destain solution. The gel fragments were dehydrated in 200µL acetonitrile followed by aspiration of the acetonitrile and dessication of the gel fragments under vacuum. The gel fragments were reduced with 50µL

10mM DTT for 30 minutes at room temperature followed by removal of the DTT solution and alkylation with 50µL of 50mM iodoacetamide for 30 minutes at room temperature. The iodoacetamide solution was removed and the gel fragments washed with 100µL of 100mM ammonium bicarbonate for 10 minutes at room temperature. The rehydration with ammonium bicarbonate solution and subsequent dehydration with 200µL acetonitrile was repeated twice. The gel fragments were dried under vacuum after which 50µL of a trypsin solution (20ng/µL porcine trypsin (Roche) freshly made in chilled 50mM ammonium bicarbonate) was added and incubated for 10 minutes on ice to rehydrate. Excess trypsin solution was removed and replaced with 20µL of 50mM ammonium bicarbonate solution after which the gel fragments were incubated at 37˚C overnight. The digested peptides were extracted by addition of 30µL of 100mM ammonium bicarbonate solution followed by vortexing, a 10 minute incubation and centrifugation at 16 000*g* for 5 minutes. The supernatant was transferred to a microcentrifuge tube. 30µL extraction solution (5% (v/v) formic acid in 50% (v/v) acetonitrile) was added to the gel fragments and incubated for 10 minutes at room temperature after which it was combined with the original supernatant and the extraction was repeated once. The extractions were concentrated under vacuum to a final volume of approximately 20µL and analysed by LC-MS-MS (Central Analytical Facilities, University of Stellenbosch, Stellenbosch).

2.2.2.19 Peptide sequencing of DDase from *Gluconobacter oxydans* ATCC 19357

Peptides were sequenced on a Waters API Q-TOF Ultima. A sample volume of 5µL was introduced at 200nL/ min by a Waters CapLC Gradient. An Atlantis dC18, 3µm, 100µmx150mm, M051511A02, column was used to separate the peptides for sequencing at a flow rate of 1.5µL/ minute with a solvent gradient of 3 to 100% Solvent B. Solvent A and B were 2% (v/v) acetonitrile, 0.2% (v/v) formic acid and 98% (v/v)

acetonitrile, 0.2% (v/v) formic acid, respectively. The gradient was developed over 60 minutes. Mass spectra were acquired for 400 to 1995 *m/z* every 0.5 seconds with a 0.1 second inter-scan delay.

Peptide sequences were analysed using Mascot (Matrix Science) using the MS/MS Ions search function. The probability based Mowse score was used as an indication of confidence in the observed match. The Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores of greater than 38 indicate identity or extensive homology ($p<0.05$).

2.2.2.20 PCR of degenerate primers designed from peptide sequences

Taking *Gluconobacter* codon usage into account, degenerate primers were designed from peptide sequences of glycosyl transferases from four bacterial species that showed high homology to sequences obtained from the tryptic digest of DDase obtained by peptide mass sequencing. The bacterial species and complementary primer sets were: *Halothermothrix orenii* H 168, *Ho*-A Fwd 5' ATC TTC GAY ACS GTS TTC GCS ATC 3', *Ho*-a Rev 5' GAT SGC GAA SAC SGT RTC GAA GAT 3'; *Geobacillus kaustophilus*, *Gk*-B Fwd 5' CAT ATC GCS GTS GAR TAT GAR 3', *Gk*-b Rev 5' YTC ATA YTC SAC SGC GAT ATG 3'; *Trichodesmium erythraeum* IMS101, *Te*-C Fwd 5' GAY TAY TGY TTC CGI CAT ATG 3', *Te*-c Rev 5' CAT ATG ICG GAA RCA RTA RTC 3' and *Thermosipho melanesiensis* BI429, *Tm*-D Fwd 5' ATC AAY GAR GCS ATG GAR ATC 3', *Tm*-d Rev 5' GAT YTC CAT SGC YTC RTT GAT 3'.

Every forward primer was used in a reaction with every reverse primer, except where large Tm differences discouraged the use of PCR analysis. Two sets of PCR reactions were done with extension reactions at 47˚C and 52˚C, respectively.

Amplification conditions for forward primers *Gk*-B, *Te*-C and *Tm*-D and the complementary reverse primers *Gk*-b, *Te*-c and *Tm*-d were: 94°C for 5 minutes; 5 x (94°C for 30 s, 40°C for 30 seconds, 72°C for 2 minutes) followed by 30 x (94°C for 30 s, 47°C for 30 seconds, 72°C for 1 minute); 72°C for 7 minutes. Amplification conditions for forward primer *Ho*-A and reverse primers *Ho*-a, *Gk*-b and *Tm*-d were: 94°C for 5 minutes; 5 x (94°C for 30 s, 45°C for 30 seconds, 72°C for 2 minutes) followed by 30 x (94°C for 30 s, 52°C for 30 seconds, 72°C for 1 minute); 72°C for 7

minutes. Bands were excised, purified with the QIAquick Gel Extraction Kit (Qiagen) as per manufacturer's instructions and sequenced (Section 2.2.2.23).

2.2.2.21 Construction of the *G. oxydans* genomic library

Genomic DNA (Section 2.2.2.17) from *G. oxydans* (ATCC 19357) was partially digested with *Sau3A* (0.2 units added to 7µg DNA in a 50µL reaction volume) at 37˚C for 1, 2, 3 and 4 minutes, respectively. The samples were heated to 80˚C for 20 minutes and separated immediately on a 0.5% (w/v) agarose gel. Fragments of between 5 and 11Kb were excised from the gel and purified using a Qiagen QIAquick Gel Extraction Kit as per manufacturer's instructions. The gDNA fragments were ligated into the ZAP Express vector using the ZAP Express Predigested Vector Kit (Stratagene; *BamHI* digested) as per the manufacturer's instructions.
Plasmid DNA was isolated by a mass excision as described by the manufacturer.

2.2.2.22 Bradford protein determinations

All protein determinations were done according to the method described by Bradford (1976). Protein concentration was determined using a commercially available protein assay solution (Bio-Rad). Bovine Serum Albumin (Fraction V) (Roche) was used as protein standard.

2.2.2.23 Sequencing

All sequencing reactions were done by the Central Analytical Facilities, University of Stellenbosch, Stellenbosch.

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Chapter 3

Results and discussion

The molecular weights of the DDase proteins of *Gluconobacter oxydans* have been estimated to be approximately 150 kDa for the extracellular isoform and 300 kDa for the intracellular isoform (Yamamoto *et al.*, 1992; Suzuki *et al.*, 2001). These estimates provide clues as to what genes within the *G. oxydans* genome may code for them. The genome of *Gluconobacter oxydans* ATCC 621H has recently been published (Prust *et al.*, 2005) and a search of the genome on the National Center for Biotechnology Information website (NCBI; http://www.ncbi.nlm.nih.gov) revealed that the largest chromosomal genes were hypothetical protein *GOX0496* (1750 aa) and a probable ATP-dependant helicase Lhr (1721 aa). The molecular weight of the hypothetical protein GOX0496 was calculated to be 182.7 kDa and contains an rfaG conserved domain sequence involved in cell envelope biogenesis (CDS, NCBI) and representing the glucosyltransferase I family of CDSs found in several *G. oxydans* glycosyltransferases (NCBI). A search of proteins of ~150 kDa revealed no further putative glycosyltransferase genes of chromosomal origin. An online search of the five *G. oxydans* plasmids revealed no glycosyltransferases or putative glycosyltransferases.

The putative DDase sequence, coding for hypothetical protein GOX0496 and ligated into the pBluescriptSK plasmid, was obtained from Dr James Lloyd, Institute for Plant Biotechnology, University of Stellenbosch. This was originally amplified by PCR from genomic DNA isolated from the *G. oxydans* strain ATCC 19357. The vector with *GOX0496* was transformed into the *E. coli* phosphoglucomutase (*pgm*) mutant. Mutants of *pgm* accumulate linear α(1→4)-linked glucans which would be used as substrate for any DDase activity. Any subsequent dextran produced, with mainly α(1→6)-linkages, would thus not colour as prominently as the α(1→4)-glucan linkages in the control when stained with iodine (Figure 1). The control bacteria stain blue/ black due to the accumulation of the α(1→4)-linked glucans while the bacteria transformed with the *GOX0496* sequence containing vector stain far less prominently, ostensibly due to fewer α(1→4)-glucan linkages and a higher proportion of linkages that do not colour with iodine. This demonstrates that the GOX0496 protein probably

utilises MOS as a substrate, converting, or possibly degrading, the MOS into another compound without or with fewer α(1→4)-glucan linkages.

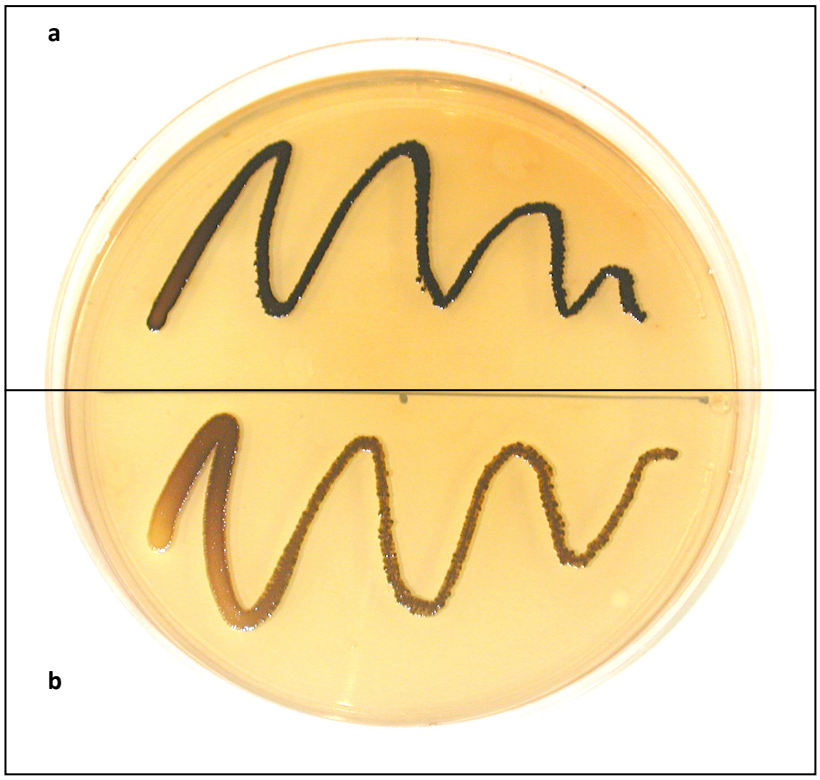

Figure 1. LBA plate with *E. coli pgm* mutants transformed with control pBluescriptSK (a) and pBluescriptSK with *GOX0496* sequence (b). The plate was supplemented with 100mM maltose and 100μg/mL ampicillin. The accumulation of linear α(1→4)-linked glucans in the control stain blue/ black with iodine while the stain is less prominent in the pBluescriptSK with *GOX0496*

The *GOX0496* sequence was amplified using specific primers for ligation into expression vectors and the amplicon separated on a 1% (w/v) agarose gel for visualisation (Figure 2). The amplicon was excised and purified using a gel extraction kit and ligated into the pGEM-T Easy vector. A restriction digest was performed using *EcoRI* restriction endonuclease which cut areas flanking the insert. This digest was separated on an agarose gel demonstrating an insert of approximately 5000 bp in length which is approximately the length of the *GOX0496* gene. In order to examine the activity of the protein, it was amplified with a second set of primers leading to the insertion of a restriction site upstream of the start codon allowing the protein to be ligated in frame with the GST tag present in the pGEX-4T-1 vector.

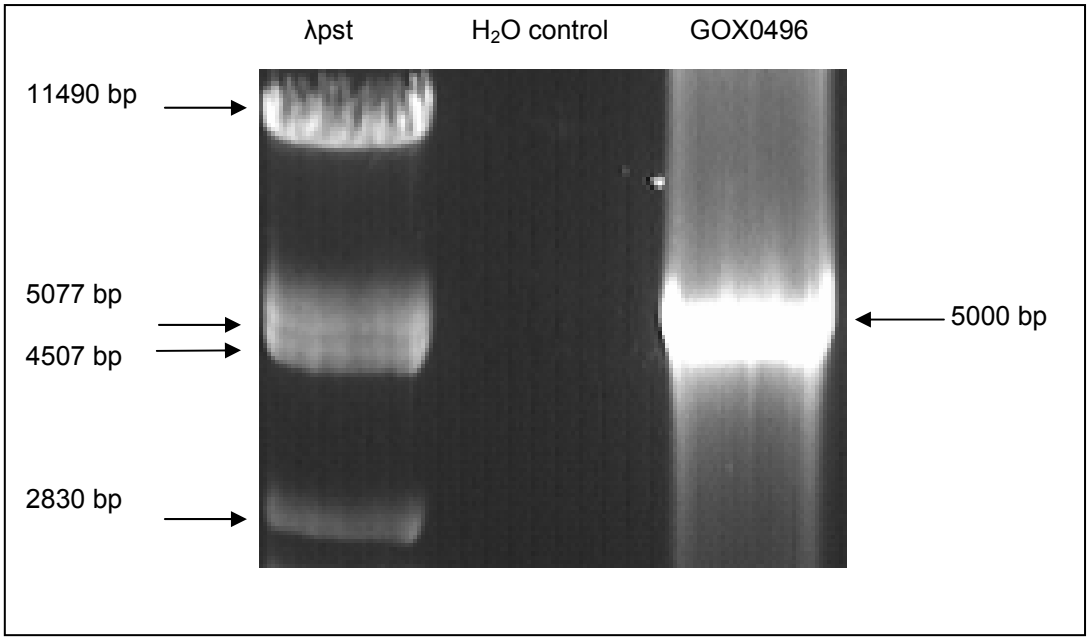

Figure 2. A 1% (w/v) agarose gel with DNA molecular weight ladder, negative template control and the PCR amplicon with the *GOX0496* sequence in the pBluescriptSK expression vector as template.

The pGEX-4T-1 bacterial expression vector was used to transform the Rosetta BL-21 strain of *E. coli* and was grown in liquid media with ampicillin. Following induction of gene expression using IPTG, the cells were lysed and the fusion protein was partially purified on a glutathione-agarose column and the isolated proteins separated on an SDS-PAGE gel (Figure 3).

In both the empty vector control and the pGEX-4T-1/ GOX0496 samples, the GST tag eluted from the column in the second elution fraction. In the control only one band was seen, corresponding to the MW of the GST tag alone, while in the cells containing the fusion protein a second, much larger band was seen.

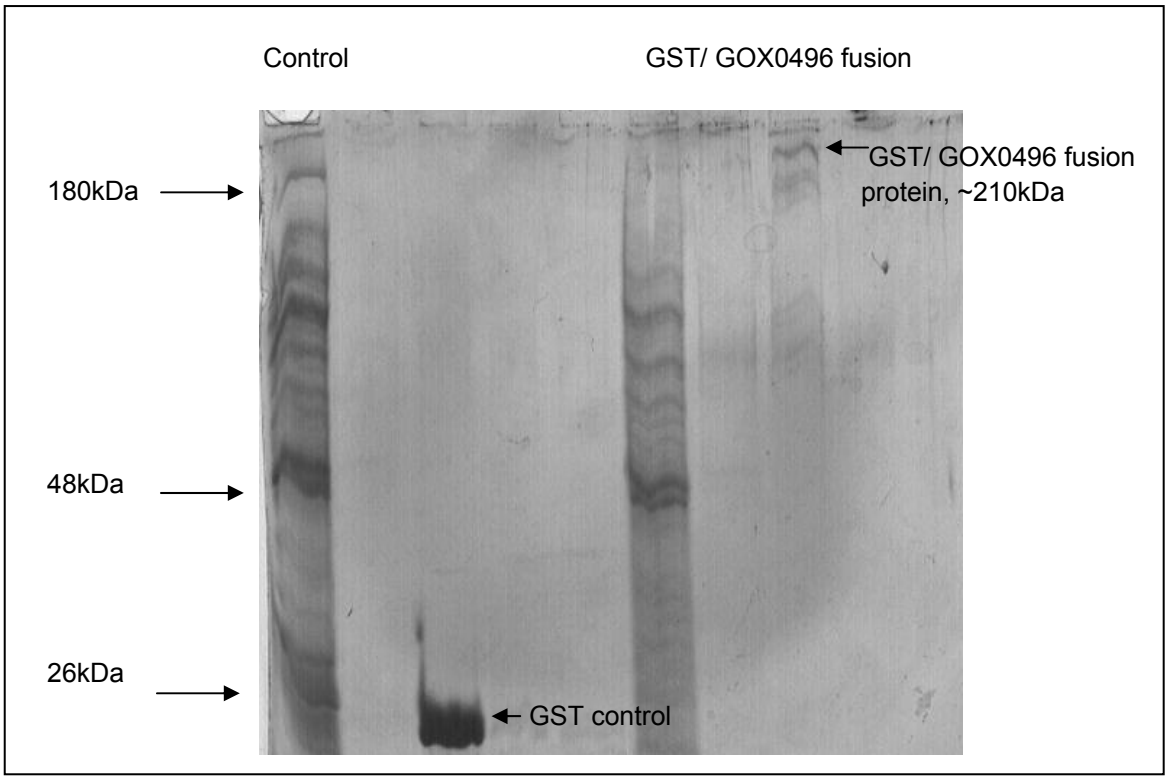

Figure 3. A 10% SDS-PAGE with molecular weight markers and four elution fractions for the empty vector control (1-4) and the GST/GOX0496 fusion protein (lane 5-8). The gel was stained with Coomassie brilliant blue.

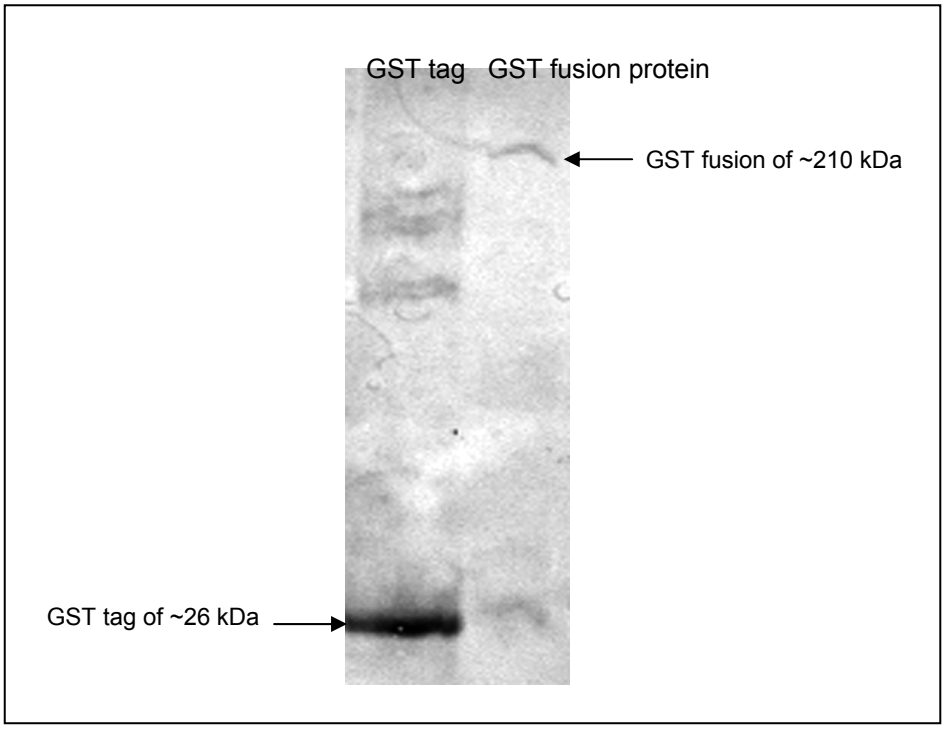

Figure 4. Immunoblot of GST tag and GST/ GOX0496 fusion proteins partially purified by glutathione agarose column. Mouse anti-GST antibody was used to probe the protein transfer after which the blot was developed with NBT/BCIP.

An immunoblot of the control GST tag and the GST/ GOX0496 fusion proteins purified by glutathione agarose columns confirmed the approximate MW of the tag and fusion protein (Figure 4). The immunoblot was probed with polyclonal mouse anti-GST as primary antibody and, after incubation with alkaline phosphatase conjugated goat anti-mouse-IgG, developed with NTB/BCIP.

Equal amounts of the GST tag and GST/ GOX0496 fusion protein were added to a 40mM solution of ρ-nitrophenyl-α-D-glucopyranoside (NPG). This has been demonstrated to be a substrate for glycosyltransferases such as DDase (Naessens *et al.*, 2003). Such transferases cleave nitrophenyl from the NPG molecule leading to production of a yellow colour. Nitrophenyl has a peak absorbance at 400nm and a molar extinction coefficient of 18 300 M^{-1}. cm^{-1}. The NPG was reacted with 5µg protein and after an hour the reaction was stopped and the absorbance over the linear phase of the reaction recorded. An activity of 258.4 and 634.7 µmol nitrophenyl. µg $protein^{-1}$. $minute^{-1}$ was calculated for the GST tag and the GST/ GOX0496 fusion protein, respectively (Figure 5).

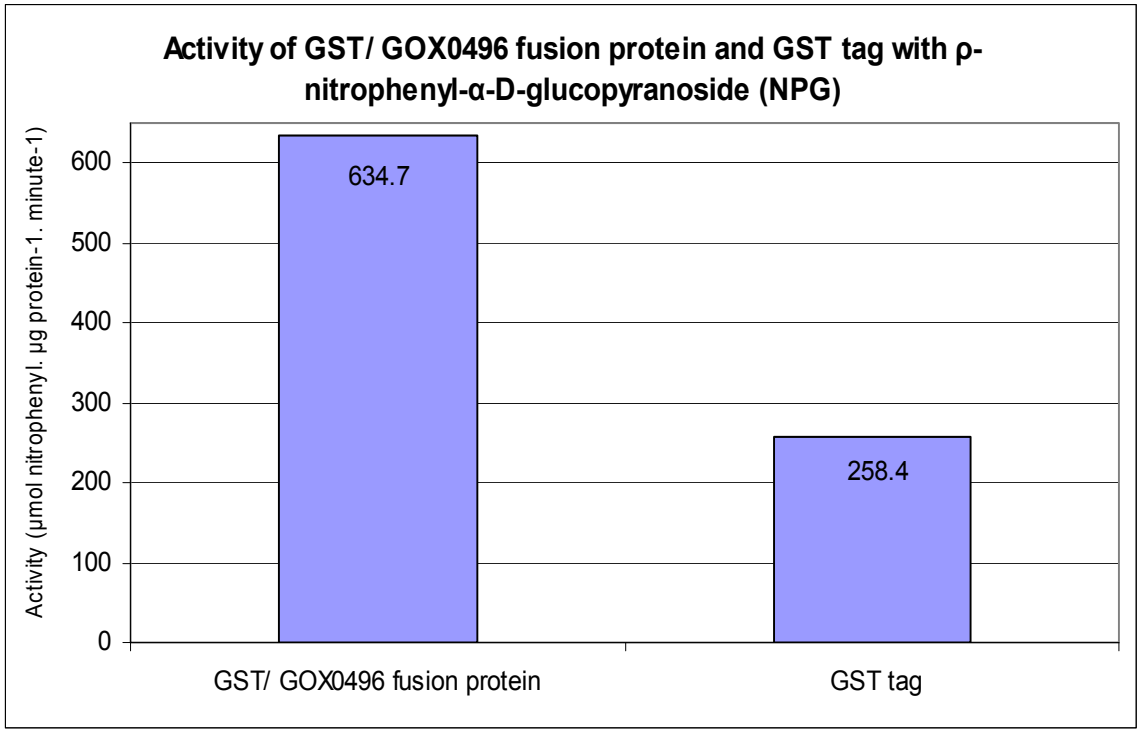

Figure 5. The activities of the GST/GOX0496 fusion protein and the GST tag. The values are given as the figure at the head of the column. The activities were calculates as µmol nitrophenyl cleaved. µg $protein^{-1}$. $minute^{-1}$.

The activity found in the control sample is possibly due to contaminating *E. coli* glycosyltransferases, however, the increase in activity in the sample containing the GOX0496 protein may be ascribed to the action of that protein. It has been seen that GOX0496 affects linear α(1→4)-linked glucan accumulation in the *E. coli pgm* mutant and contains a transferase CDS. DDase has been reported to manufacture dextran from a solution of maltotriose (Suzuki *et al.*, 2001), thus activity of the GST/ GOX0496 fusion protein was assessed using maltotriose as a substrate. No activity of the protein could be found using maltotriose as a substrate (Data not shown since no dextran was produced), probably because the GOX0496 protein does not code for DDase, but rather some other glycosyltransferase (See results below).

Taken together these data would indicate that although *GOX0496* codes for a glycosyltransferase that utilises MOS, it does not code for a protein with DDase activity. This is shown as expression of the gene in the *pgm* mutant of *E. coli* leads to a change in the accumulation of linear α(1→4)-linked glucans (as shown by a change in colouration when stained with iodine), but the partially purified protein is unable to produce dextran from maltotriose. It is possible that the protein is able to utilise linear α(1→4)-linked glucans to produce some other polyglucan, but that remains to be investigated. The *G. oxydans* genome strain that was sequenced (Prust *et al.*, 2005; ATCC 621H) was not utilised for amplification of the *GOX0496* gene. The genome sequence data of ATCC 621H was used to search for DDase based on gene size, however, the strain has never been shown to produce DDase. It was decided to compare strain ATCC 621H with ATCC 19357 to compare differences in this respect. To this end the ATCC 621H strain was obtained from the DSMZ stock center (http://www.dsmz.de/).

Both the ATCC 19357 and ATCC 621H strains were grown in liquid culture and cell free extracellular proteins partially purified by centrifugation as described previously (Chapter 2, Section 2.2.2.1). Activity of *G. oxydans* ATCC 19357 DDase was calculated as dextran production using maltotriose as a substrate and compared to the results of Suzuki *et al.,* 2001 (Table 1). Because the protein from the centrifugation step was resuspended in 5mL of buffer as opposed to the 117mL described by Suzuki *et al.,* 2001, total protein and total activity were multiplied by the factor of the difference after the resuspension of the protein in buffer. Similar values for protein mass, total activity and thus specific activity were obtained.

Table 1. Comparisons of experimental results of this study to that of Suzuki *et al.*, 2001. The total and specific activities are compared after the volumes of resuspended protein obtained after centrifugation are normalised to that reported by Suzuki *et al.*, 2001.

		Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)
Reported in literature (Suzuki *et al.*, 2001)	Centrifugation	117	11409	20.5	702.9
	Dialysis	125	11409	20.5	702.9
Experimental results from *G. oxydans* ATCC 19357	Centrifugation	5	557	0.7	795.4
	Normalised volume	117	13034	16.4	795.4

Activity gel analysis of the partially purified proteins obtained from the cell-free cultures of *G. oxydans* ATCC 19357 and ATCC 621H using PAGE and Schiff staining resulted in clearly discernable purple staining bands in lanes loaded with protein from the ATCC 19357 culture (Figure 6). The purple colouring is the result of the Schiff reagent reacting with aldehyde groups formed by glucosidic residues being selectively oxidized by periodic acid. The purple band is thus localised polyglucan formation after incubation with MOS. No staining was observed in lanes loaded with protein from ATCC 621H. The staining bands correspond in position on the gel to the cell-free extracellular proteins seen on the SDS-PAGE when comparing expressed proteins from strains ATCC 19357 and ATCC 621H.

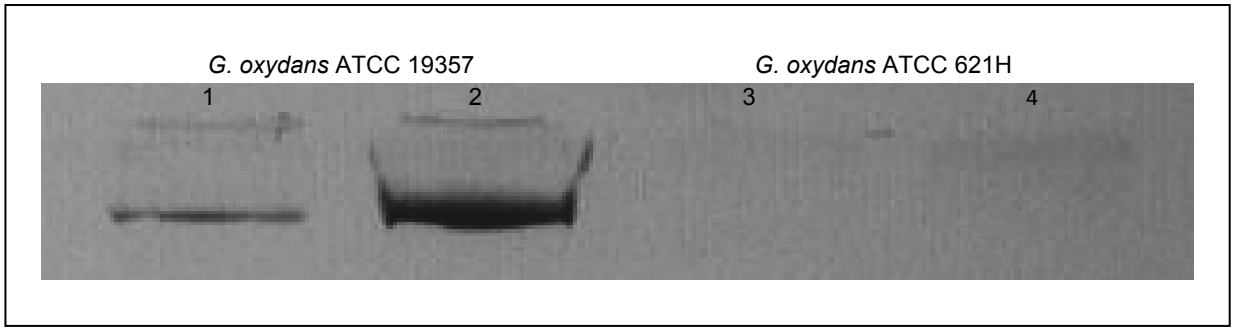

Figure 6. A 6% native gel with protein from cell-free cultures of ATCC 19357 and 621H. 0.4µg and 1.7µg partially purified protein from ATCC 19357 was loaded onto lanes 1 and 2, respectively, while equal masses of partially purified protein from strain ATCC 621H was loaded into lanes 3 and 4. Activity gels were cast without SDS and the proteins separated at 4°C. SDS was omitted from the running buffer. The gels included MOS and dextran as primer. Schiff reagent stains aldehyde groups, produced by the oxidation of glucose residues, purple.

To demonstrate that the band stained by the Schiff reagent is dextran an experiment was performed where the gel was first incubated with protein from strain ATCC 19357 and MOS, and then in dextranase (an enzyme which degrades dextran). The polyglucan was then detected with Schiff staining. Gels incubated only with the substrates demonstrated a band of polyglucan in lanes where protein from the ATCC 19357 strain was applied (Figure 7, lanes 1 and 2), but not those where protein from the ATCC 621H strain (Figure 7, lanes 3 and 4) was loaded. The band produced by the protein from the ATCC 19357 strain disappeared when incubated with dextranase (Figure 7, lanes 5 and 6), thus demonstrating that the band is dextran.

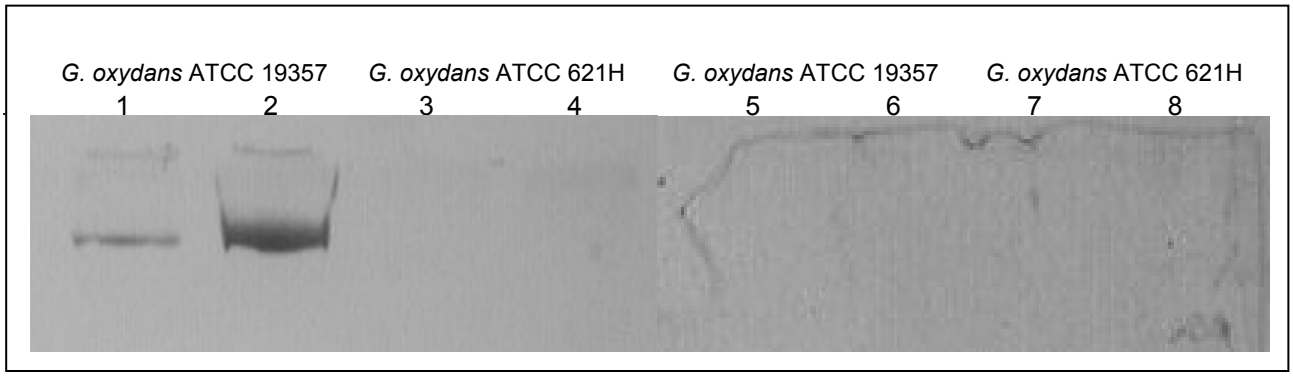

Figure 7. Two identical 8% activity gels were incubated with 1mg/ml MOS after separation. The right gel (lanes 5 – 8) was then incubated with 150 units of dextranase in 50mM sodium acetate buffer; pH 6.0, overnight. Both gels were developed with the Schiff staining method. Lanes 1, 2, 5 and 6 were loaded with partially purified protein from strain ATCC 19357 while lanes 3, 4, 7 and 8 were loaded with partially purified protein from strain ATCC 621H. 1.7µg of the partially purified protein was loaded into lanes 2, 4, 6 and 8 and 0.4µg in lanes 1, 3, 5 and 7.

Proteins were separated on a 10% (v/v) SDS-PAGE to compare differential protein expression between the strains (Figure 8). Previous experiments have demonstrated that *G. oxydans* strains which produce the extracellular DDase ($DDase_{ext}$) secrete a protein of 152 kDa (Suzuki *et al.*, 2001). *G. oxydans* ATCC 19357 displayed two prominent protein bands of approximately 150 and 50 kDa when stained with Coomassie dye while ATCC 621H displayed only the smaller band. The larger protein corresponds in size to the extracellular DDase reported in literature, and given that the ATCC 621H strain does not show DDase activity in native gels while the ATCC 19357 strain does it can be assumed that it is the $DDase_{ext}$.

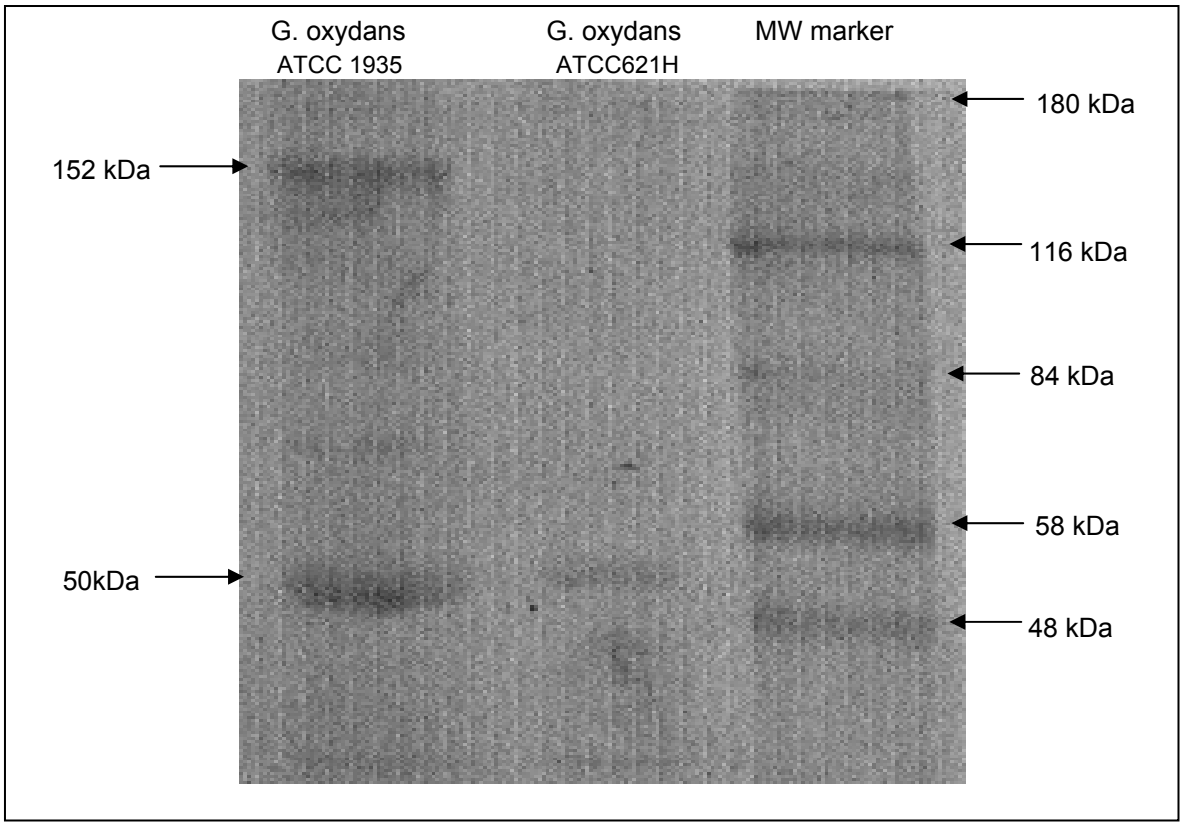

Figure 8. A 10% (v/v) SDS-PAGE of the extracellular proteins from *G. oxydans* ATCC 19357 and *G. oxydans* ATCC 621H. *G. oxydans* ATCC 19357 is shown as having two prominent proteins of 152 kDa and 50 kDa while *G. oxydans* ATCC 621H displays only the 50 kDa protein.

DDase extends the dextran molecule using MOS as substrate. When maltotriose alone is utilised it leads to the liberation of maltose, while glucose is transferred to the growing dextran polymer. Maltose too is acted upon by DDase, producing glucose and the trisaccharide panose. The increase of glucose and panose in a reaction mixture containing DDase can be used to gauge relative DDase activity. However, because of the disproportionation actions of DDase (where DDase may form α(1→4)- instead of α(1→6)- linkages and may hydrolyse α(1→6)- linkages with subsequent reformation of the α(1→6)- linkage) a direct correlation between activity and glucose or panose release is difficult to make. The glucose released into solution was measured when cell-free, partially purified protein was incubated with maltotriose for one hour. The value shown is the percentage of glucose in solution against total glucose in the form of substrate maltotriose (Figure 9). Nearly thirty times more glucose was found in solution when maltotriose was incubated with extracellular protein from *G. oxydans* ATCC 19357 than with ATCC 621H. The glucose found in solution after incubation with strain ATCC 621H could be from residual glucose in the maltotriose substrate.

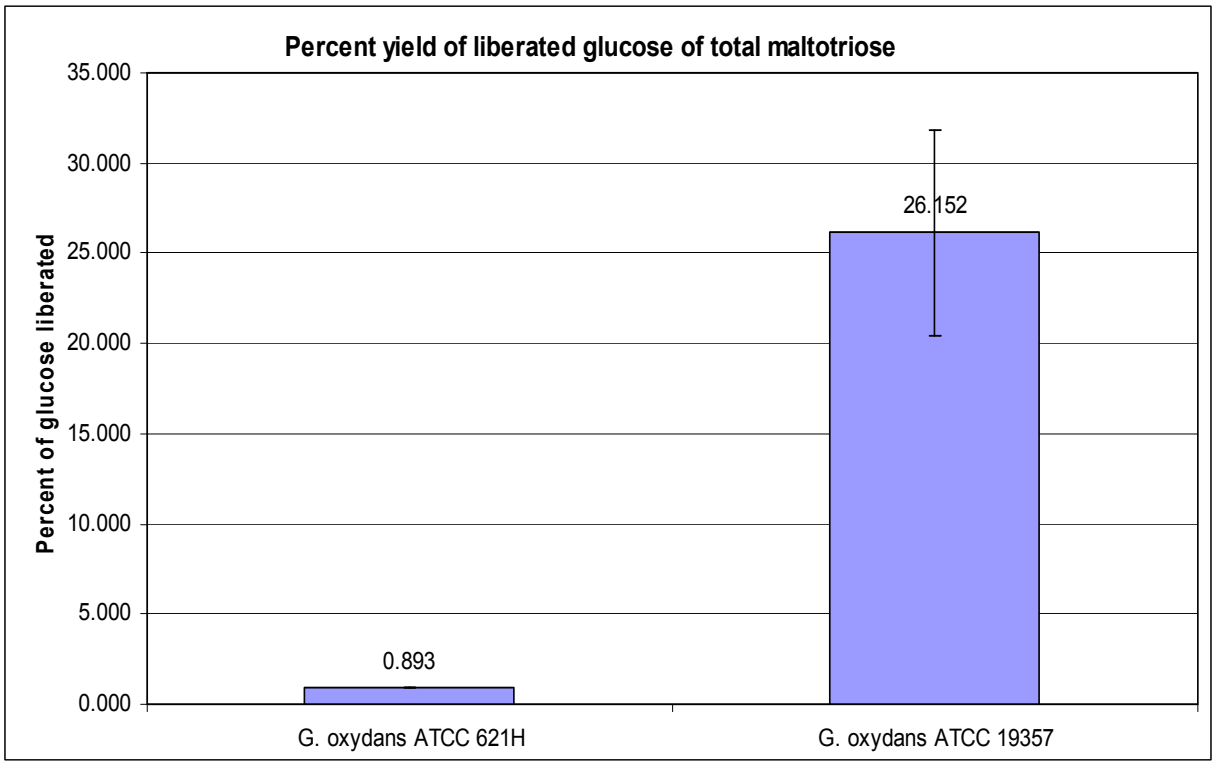

Figure 9. Production of glucose from maltotriose for two strains of *Gluconobacter oxydans,* ATCC 621H and ATCC 19357. The values represent the mean percent of total glucose cleaved from polymeric molecules in solution. Samples were performed in triplicate and the error bars represent standard errors.

DDase produces dextran polymer when incubated with maltotriose (Suzuki *et al.*, 2001). To examine whether dextran was produced when the cell free extracts from the two *G. oxydans* strains were incubated with maltotriose, any polymers produced during such an incubation were isolated by precipitating them using ethanol. The pellets produced by such precipitations were then re-suspended in water and digested either with HCL, which should hydrolyse all linkages between glucose units within the polymer, or dextranase which digests dextran to isomaltose. The products of these digests were separated by thin layer chromatography (TLC) and compared with glucose and maltose standards. Sugars on the TLC plates were visualised by application of ρ-anisaldehyde reagent and colour development at 100°C (Figure 10).

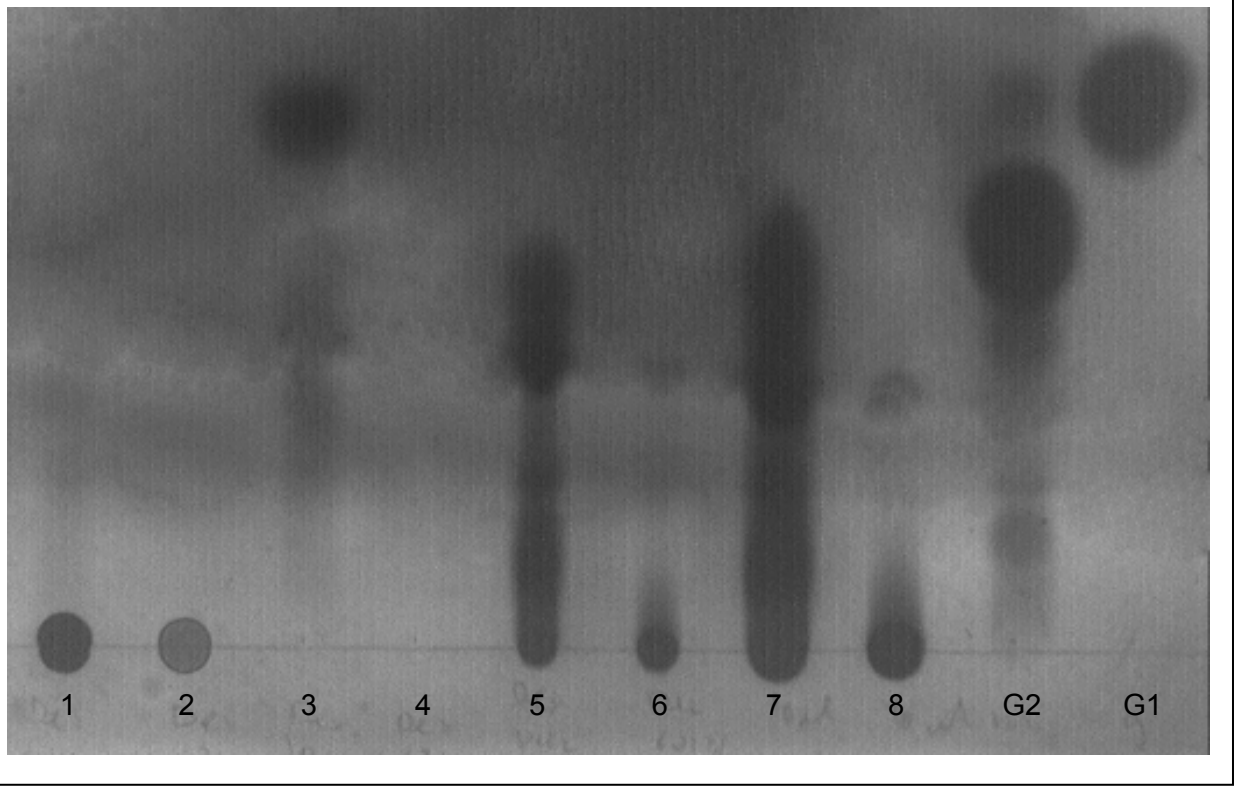

Figure 10. TLC of hydrolysis products of dextran produced from partially purified protein from *G. oxydans* strains ATCC 19357 and ATCC 621H. Lanes 1 and 2: reaction products of ATCC 19357 and 621H with maltotriose, respectively. Lanes 3 and 4: digestion of 5µL of the reaction products of ATCC 19357 and 621H with HCl; 5 and 6: digestion of 5µL of the reaction product with dextranase. Lanes 7 and 8: digestion of 10µL of the reaction product with dextranase. Samples G2 and G1 are maltose and glucose standards, respectively.

In figure 10 it can be seen that the dextran pellet isolated from the incubation before digestion (lanes 1 and 2) does not migrate from the loading point. Acid hydrolysis led to the production of glucose from the pellet isolated following incubation of MOS with the extract from the ATCC 19357 strain (lane 3), but not the ATCC 621H strain (lane 4). Incubation with dextranase led to the production of a sugar which runs to a similar point on the plate as maltose and which is probably isomaltose. Again this was only seen in the pellet produced by extracts from the ATCC 19357 strain (lanes 5 and 7) and not the ATCC 621H strain (lanes 6 and 8). This provides further evidence that the ATCC 19357 strain secretes DDase while the ATCC 621H does not.

Dextran produced by the incubation of the partially purified protein from *G. oxydans* strain ATCC 19357 was digested with dextranase from *Penicillium* sp. and α-

glucosidase from *S. cerevisiae* and compared to the same digest of a commercially available dextran isolated from *L. mesenteroides* (Figure 11).

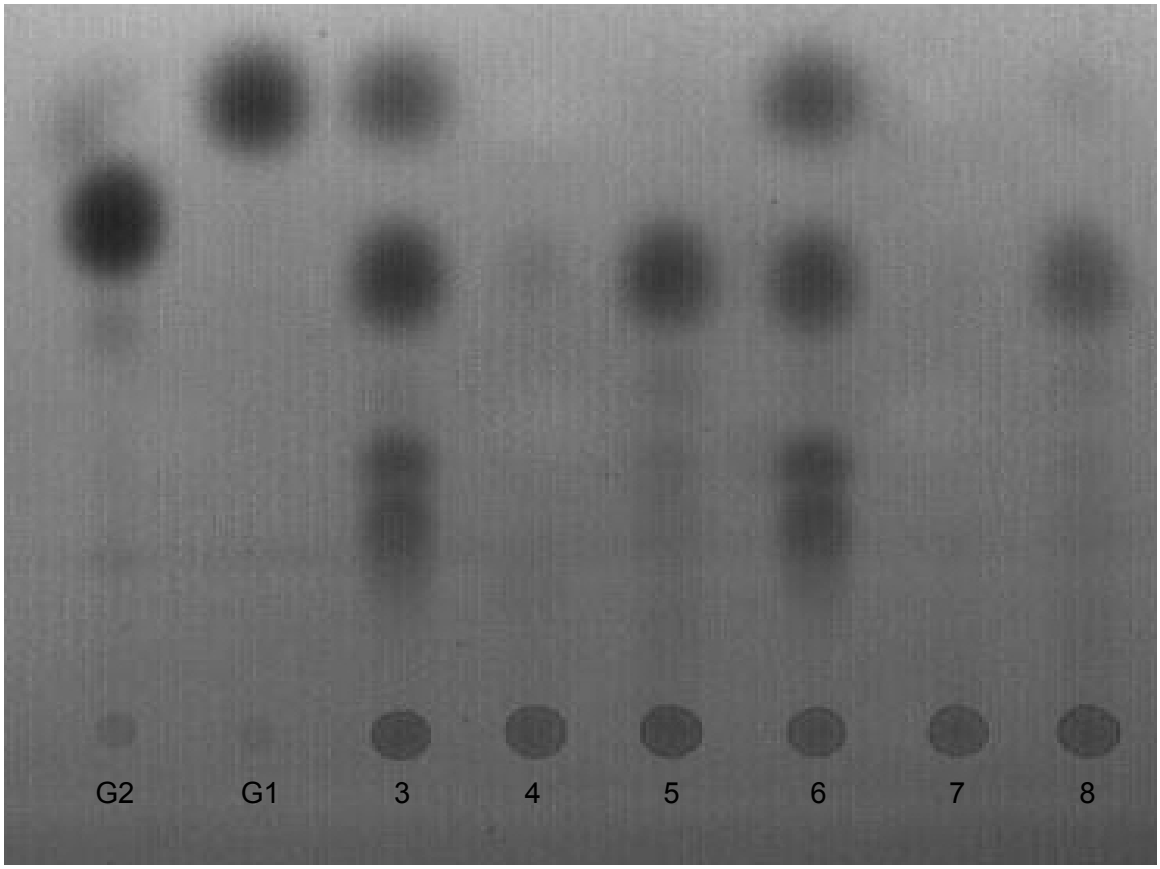

Figure 11. TLC of digestion products of dextran from *L. mesenteroides* and dextran produced from DDase using maltotriose and MOS and enzymatic digestions thereof. Lanes G2 and G1: Maltose and glucose, respectively. Lane 3, 4 and 5: 1% (w/v) lab stock *L. mesenteroides* dextran and dextran produced *in vitro* from 1mg/mL maltotriose and MOS, digested with dextranase from *Penicillium* sp, respectively. Lane 6, 7 and 8: 1% (w/v) lab stock *L. mesenteroides* dextran and dextran produced from 1mg/mL maltotriose and MOS, digested with dextranase and α-glucosidase from *S. cerevisiae* (Type1), respectively.

In figure 11 dextran from three sources has been digested with different enzymes. In lanes 3, 4 and 5 are respectively dextran from *L. mesenteroides* and dextran produced by the action of the cell free extract from *G. oxydans* ATCC 19357 on maltotriose or MOS. These dextrans have been digested with dextranase, which should lead to the production of isomaltose. A spot that migrates similarly to maltose, presumably isomaltose, was present in the dextrans manufactured by the protein extract from the ATCC 19357 strain, as well as from the commercially produced dextran. Digestion of

the *L. mesenteroides* dextran also produced other spots that are probably glucose and MOS. To examine whether the compound produced really is isomaltose, the samples were digested further with α-glucosidase from *Saccharomyces cerevisiae.* This hydrolyses isomaltose to glucose. The samples were loaded on the TLC plate in the same order as for the dextranase digest, but in lanes 6, 7 and 8. Some glucose production was noted in the digest of the dextranase produced from MOS (lane 8), but in the other two lanes it was not clear, either due to a lack of sensitivity, or to glucose being present in the sample prior to digestion with α-glucosidase, leading to a masking of any glucose production.

The K_m value for maltotriose of the partially purified DDase was calculated using a stopped reaction with substrate concentrations varying between 1 and 25mM maltotriose (Figure 12). The measured activity of the enzyme at the different concentrations was calculated and plotted against the substrate concentration. The K_m was calculated to be 6.9mM using SigmaPlot using a two-parameter rectangular hyperbola to obtain a hyperbolic curve. A previous estimate of the K_m of $DDase_{ext}$ was 10.2mM (Suzuki *et al.*, 2000), similar to the calculated K_m in this experiment. It is also reported in literature that maltose has an inhibitory effect on DDase (Suzuki *et al.*, 2000). It was not mentioned at which concentration maltose became inhibitory nor did the researchers mention the concentration range over which the K_m in their study was calculated. Thus the maltose produced by the action of DDase on maltotriose may have an inhibitory action and might have influenced the calculated figure here.

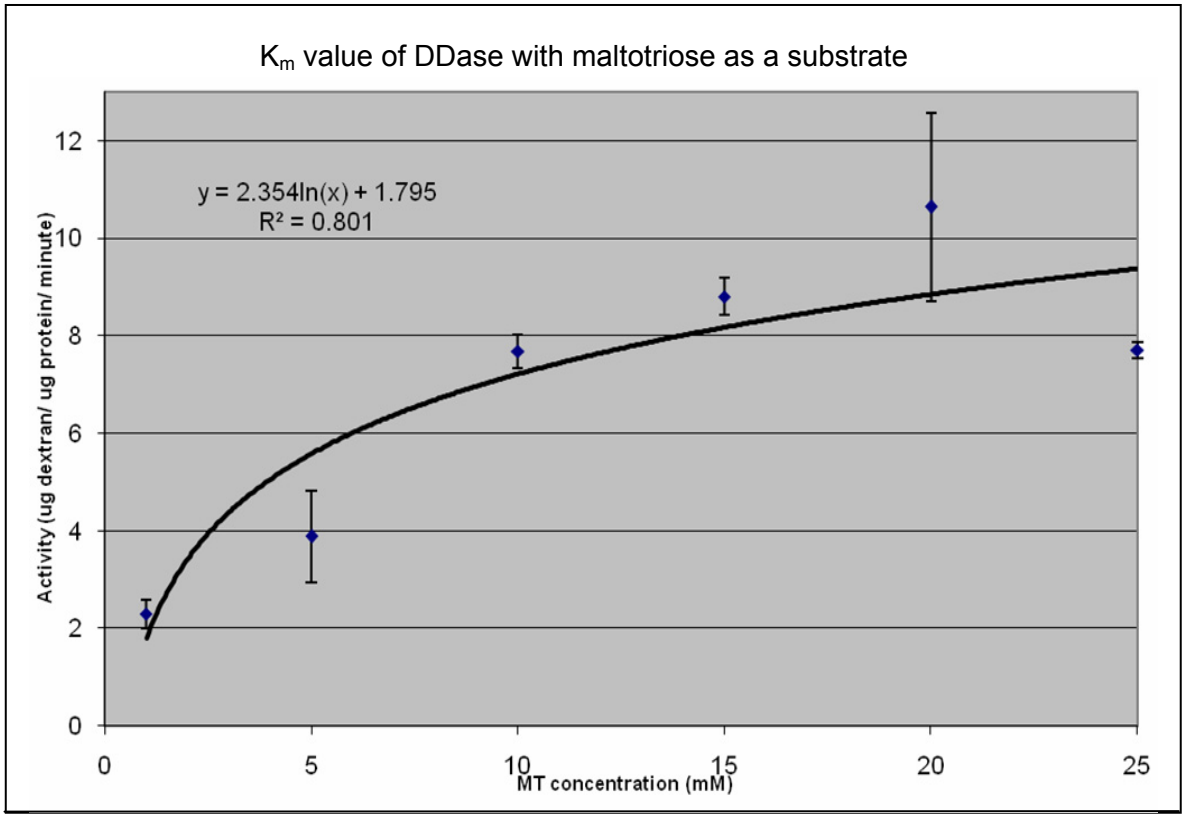

Figure 12. Graph of DDase activity (µg dextran/ µg protein/ minute) at varying substrate concentrations with maltotriose as a substrate. All data points were in triplicate. The information on the graph was used to calculate the K_m of DDase.

To see if the 150 kDa protein seen in the cell-free extract of the ATCC 19357 strain codes for a glycosyltransferase it was separated from other proteins in the extract by SDS-PAGE, and digested with trypsin. Peptides from the tryptic digest were then sequenced by LC-MS-MS. This revealed no peptide sequences similar to known *G. oxydans* proteins; however, close matches to glycosyltransferases from four separate bacterial species were found (Table 2).

Table 2. Peptide sequence matches from a tryptic digest. Peptide sequences obtained through tryptic fingerprinting were analysed on Mascot and several high homology glycosyltransferases from four separate species were found. Included are the peptide sequences from the organism and the gene to which homology was found.

Tryptic digest peptide sequence homology to glysosyltransferases (Mascot, www.matrixscience.com)		
peptide sequence	organism	gene
K.SAAAVTIIVPLLALGVPIFDTVFAIIR.R	*Halothermothrix orenii* H 168	Glycosyltransferase, family 4
R.HIAVEYEREPR.L	*Geobacillus kaustophilus*	Glycosyltransferase
R.LISDYCFRHMSNPTQDHLNK.E	*Trichodesmium erythraeum* IMS101	Glycosyltransferase, family 2
K.GEKGKPQSINEAMEIAK.G	*Thermosipho melanesiensis* BI429	Glycosyltransferase, family 2

The significant matches were for glycosyltransferases from *Halothermothrix orenii* H168, *Geobacillus kaustophilus, Trichodesmium erythraeum* IMS101 and *Thermosipho melanesiensis* BI429.

To amplify the gene coding for these sequenced peptides the sequences were used to design degenerate sense and antisense primers. PCR using each combination of sense and antisense primers was performed, thus maximising the possibility of amplification of the DDase transglucosidase. The *H. orenii* H168 sense primer was designated uppercase 'A' and the antisense a lowercase 'a'. By extending this nomenclature *G. kaustophilus, T. erythraeum* IMS101 and *T. melanesiensis* BI429 sense and antisense primers were designated 'B', 'C', 'D' and 'b', 'c', 'd', respectively.

Using gDNA isolated from strain ATCC 19357 as template, PCR led to the amplification of several fragments (Figure 13 and 14). The amplicons of the PCR reactions were excised, purified from the agarose gel, ligated into a pGEM-T Easy transformation vector and sequenced. The sequences were found to not code for transglucosidases (Table 3).

Table 3. Sequence analysis results of 7 amplicons amplified using gDNA from *G. oxydans* ATCC 19357. The sense and antisense degenerate primer sequences were derived from high homology glycosytransferase sequences from *H. orenii* H168, *G. kaustophilus, T. erythraeum* IMS101 and *T. melanesiensis* BI429.

	Sequence	Blast hit on query sequence (NCBI)
1	Bc1	Hypothetical membrane-spanning protein UPF0078 GOX1265 [*G. oxydans* 621H]
2	Bc2	Putative oxidoreductase [*Gluconobacter oxydans* 621H]
3	Bd1	Hypothetical protein GOX2494 [*Gluconobacter oxydans* 621H]
4	Dc1	Hypothetical protein GOX2047 [*Gluconobacter oxydans* 621H]
5	Aa1	Hypothetical transaminase protein GOX1103 [*Gluconobacter oxydans* 621H]
6	Aa2	*Gluconobacter oxydans* 621H, complete genome
7	Aa3	*Gluconobacter oxydans* 621H, complete genome

The seven amplicons did not sequence for any glucosyl- or glycosyltransferases as can be seen when compared to the list of all the transferases within the *G. oxydans* ATCC 621H genome (Chapter 1, Table 3.2)

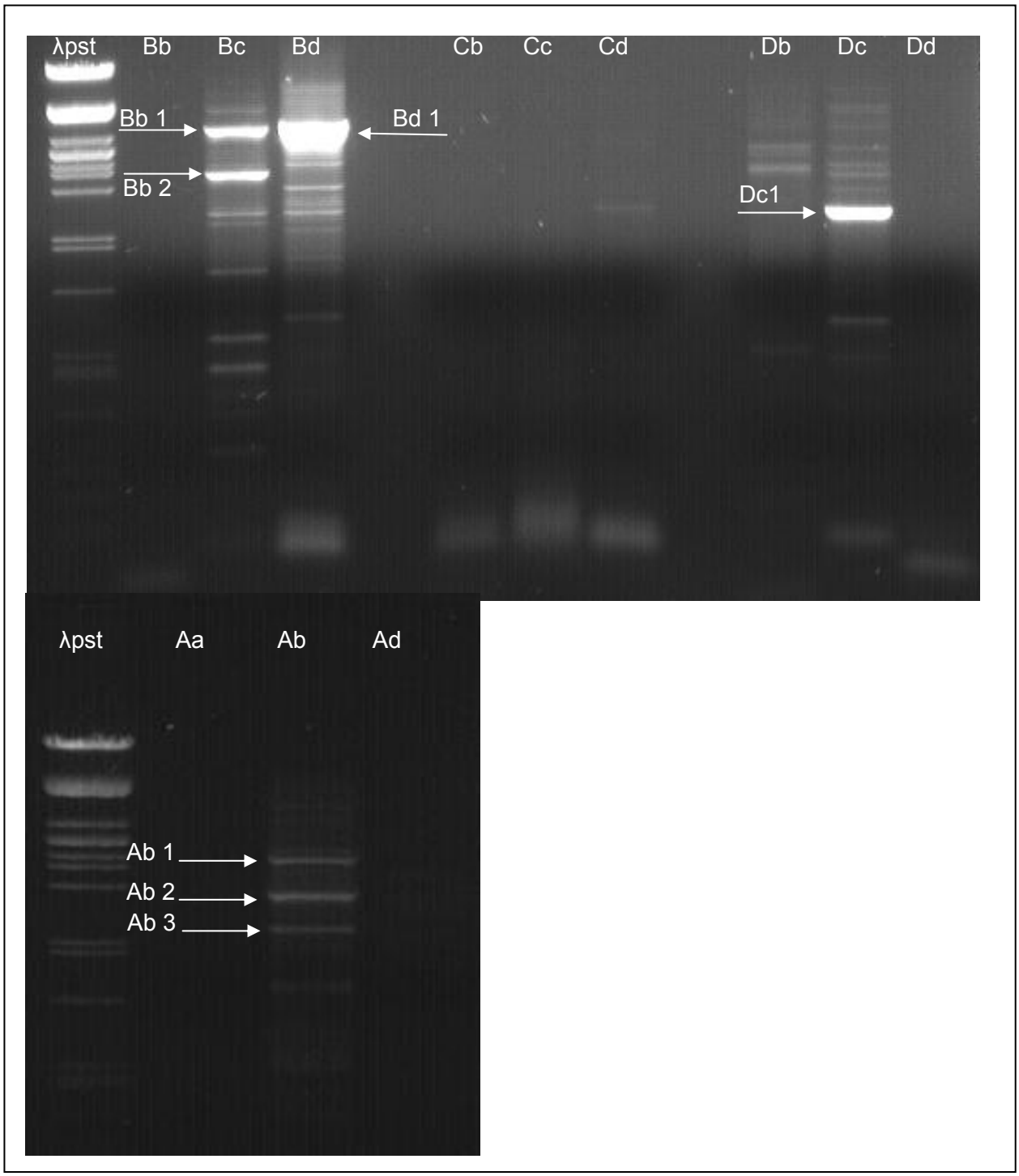

Figure 13 and 14. Four sets of degenerate primers with were used to amplify regions of *G. oxydans* ATCC 19357 gDNA. The degenerate primers were designed from sequences of transglucosidases from 4 separate species with high peptide sequence homology to DDase. Each sense primer was used with every antisense primer except where large Tm differences discouraged the use of PCR analysis. The primer set used is identified by letters; capital letters denote sense primers while lower case letters denote antisense primers. Arrows indicate bands that were excised and sequenced.

In a further attempt to isolate the DDase gene a genomic DNA library was manufactured using *G. oxydans* ATCC 19357 gDNA. Genomic DNA was partially digested for different times with the *Sau3A1* restriction endonuclease (Figure 15).

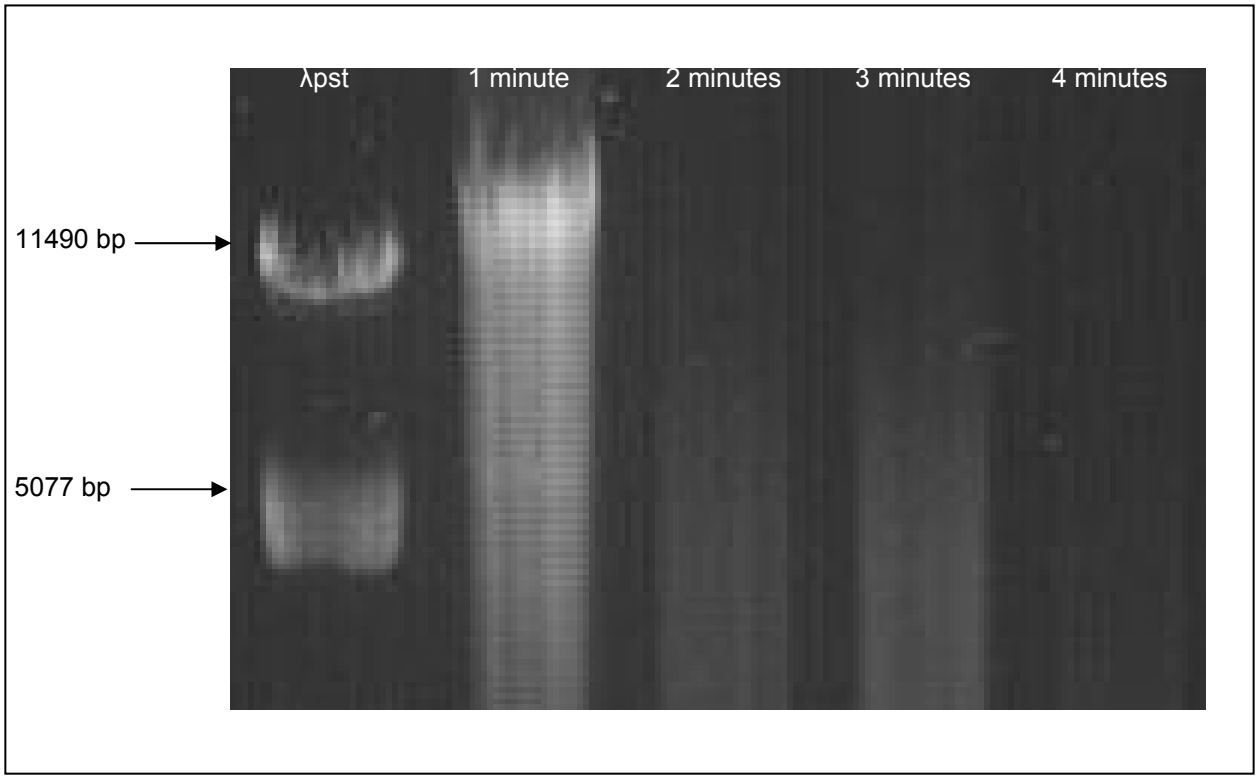

Figure 15. Partial digestion of *G. oxydans* ATCC 19357 gDNA with 0.2 units of *Sau3A1* after 1 to 4 minutes at 37°C. Approximately 50µL of a 140ng/µL gDNA solution was digested after which the DNA was separated on a 0.5% (w/v) agarose gel.

The digests were separated on a gel and fragments between 5 and 11 Kb were excised and purified. A sample of the fragments was then separated on an agarose gel to check the sizes of the fragments (Figure 16).

The fragments were ligated into the *BamHI* site of the ZAP Express phagemid. The primary library was amplified leading to a library with a titre of approximately $1.9x10^{11}$pfu/mL. Addition of X-gal into the media led to an estimate that 0.3% of the phage did not contain an insert.

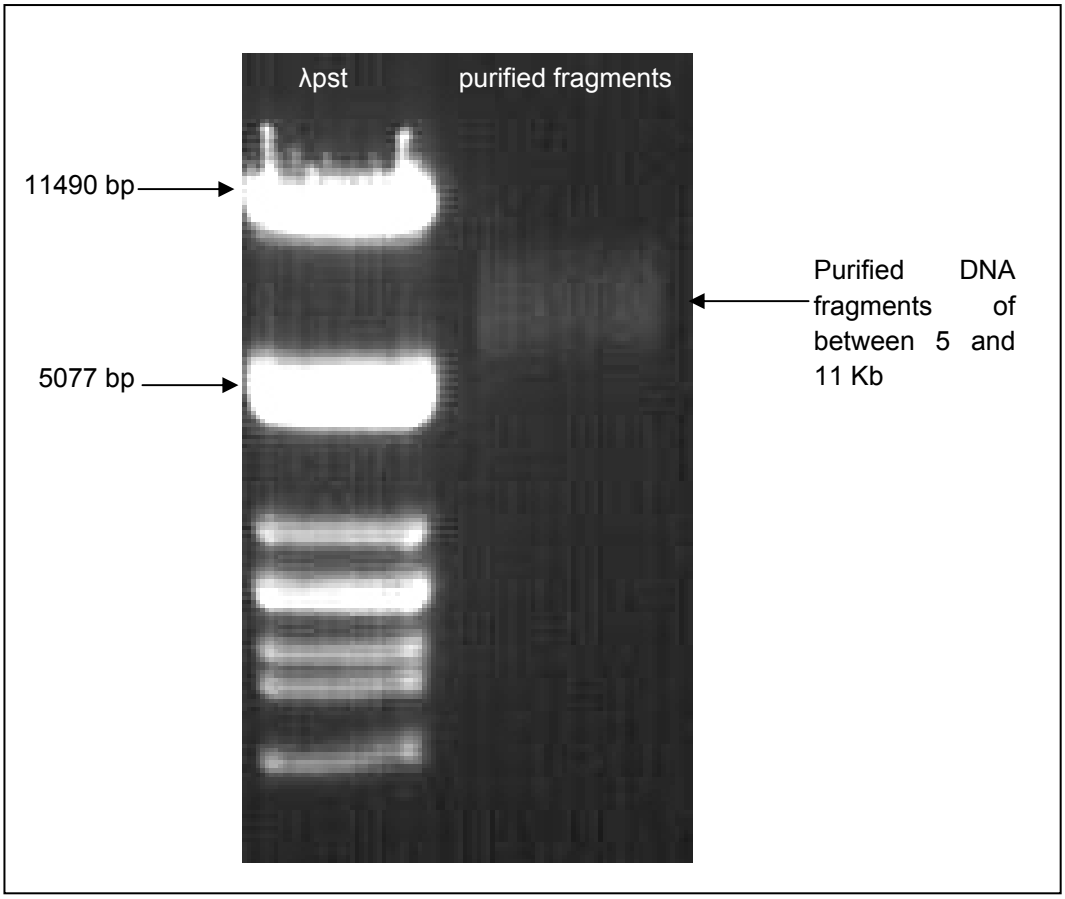

Figure 16. DNA fragments digested with *Sau3A1* and of between 5 and 11 Kbp are visualized on a 1% (w/v) agarose gel

The phage library was excised to produce a plasmid library vector and this was transformed into the *pgm* mutant of *E. coli*. The transformed bacteria were grown on media supplemented with maltose, and following growth was stained with iodine. Under these conditions the *E. coli* would stain blue due to the large amounts of MOS. These would act as a substrate for DDase which would produce dextran that does not stain as intensely with iodine. Colonies that did not stain dark blue were isolated and were partially sequenced with the T3/ T7 primer set. The ends of the inserts were sequenced, but did not lead to the identification of a glycosyltransferase gene (Table 4).The average insert size of the plasmids was approximately 7.6 Kbp as determined by digestion with the *BamHI* restriction enzyme.

Table 4. Ten colonies of *pgm* mutants, transformed with the amplified plasmid library, that stained lightly with iodine were selected and sequenced. The sequence blast result on the NCBI website is recorded as well as the number of bp of the sequence and E value. Samples indicating N/A had poor sequencing results and could not be identified.

	Sequence blast results			
	Sequence	Blasted bp	Blast hit on query sequence (NCBI)	E value
1	A10_15	1132	*Gluconobacter oxydans* 621H, complete genome	0.0
2	B09_1	1206	*Gluconobacter oxydans* 621H, complete genome	0.0
3	B10_16	1205	*Gluconobacter oxydans* 621H, complete genome	0.0
4	C09_2	1167	*L. lagopus* AC microsatellite, locus LLSD2	7.00E-30
5	C10_20	1155	*Gluconobacter oxydans* 621H, complete genome	0.0
6	D09_4		N/A	
7	E09_5	1167	*Gluconobacter oxydans* 621H, complete genome	0.0
8	F09_9		N/A	
9	G09_10	1189	*Gluconobacter oxydans* 621H, complete genome	0.0
10	H09_12	1174	*Gluconobacter oxydans* 621H, complete genome	0.0

The possibility exists that the DDase gene lies up- or downstream of these sequence results, but it was not possible to investigate this further due to time restraints.

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7. www.matrixscience.com

Chapter 4

Conclusion

The data from this study indicate that two *G. oxydans* strains are genetically different in terms of coding for the extracellular DDase enzyme. The ATCC 19357 strain secretes the DDase protein into the growth media, while the ATCC 621H strain does not. Because the genome of the 621H strain has been sequenced, the number of glycosyltransferases present in it can be estimated by sequence comparison. The only gene coding for a protein large enough to be the DDase is the GOX0496 hypothetical protein, but following its purification no DDase activity could be detected. This protein, therefore, probably has some other glycosyltransferase activity. It might be that the protein utilises MOS with more glucosyl units than maltotriose as no activity of the purified protein could be detected when incubated with maltotriose. However, when the protein is expressed in the *E. coli pgm* mutant it seems to reduce the amount of MOS which accumulates in the bacteria. It cannot be ruled out, however, that the DDase is present in the ATCC 621H genome, but shows no homology to known glycosyltransferases and is not expressed at the same time and under the same conditions as in the ATCC 19357 strain.

To try and identify the DDase gene coding for the secreted isoform from the ATCC 19357 strain, the protein was partially purified from the culture medium. *De novo* peptide sequence was obtained from the protein that probably represents the $DDase_{(ext)}$ after digestion with trypsin. Some of the peptides showed homology to glycosyltransferases from bacterial species, but not to any gene from the ATCC 621H genome sequence. These peptides are probably part of the DDase protein and were used to design degenerate primers for amplification of a fragment of the DDase gene. Unfortunately they were not successful in amplifying the gene fragment. Time restraints did not allow optimization of amplification conditions, so the primers might still be used successfully in future under other amplification conditions.

Another attempt to isolate the DDase gene involved manufacturing a gDNA library from the gDNA of the ATCC 19357 strain. A plasmid form of this library was transformed into the *E. coli pgm* mutant and was grown on maltose containing plates. If the DDase protein is expressed in these transformant *E. coli* it should utilise the linear α(1→4)-linked glucans which they manufacture, and the cells should no longer stain blue with iodine. Plasmids were isolated from colonies that did not stain blue and, although they all contained *G. oxydans* DNA, the ends of the sequences did not code for any obvious glycosyltransferases. These plasmids are currently being analysed further to see if the DDase gene would start coding in another part of the amplicons.