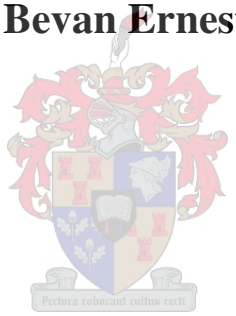
Molecular studies of galactan biosynthesis in red algae

Stanton Bevan Ernest Hector



Dissertation presented in fulfilment for the Degree of Doctor of Philosophy in Plant Biotechnology at the University of Stellenbosch

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Abstract

Sulfated galactans (agarans and carrageenans) are accumulated in the cell wall of various red algae (Rhodophyta) species. These polysaccharides are of commercial importance in the food, pharmaceutical and biotechnology industries due to their unique physicochemical properties. Although having received significant research attention over the last 20 years, events regarding their biosynthesis have not been elucidated. Aiming for the identification of galactosyltransferase (GalT) genes involved in sulfated galactan biosynthesis, cDNA expression libraries were constructed from the prolific agar-producing South African red seaweed *Gelidium pristoides* (Turner) Kützing and screened by functional complementation of UDP-galactose 4-epimerase deficient mutants (*E. coli* and *S. cerevisiae*). Regretfully, no GalTs were identified. The study however yielded the first UGE enzyme described for a red seaweed. Southern hybridization indicated the presence of two UGE copies and confirmed the gene originated from *G. pristoides*. Bioinformatic analysis of *G. pristoides* UGE shows amino acid sequence homology to known UGEs from various organisms. The enzyme was shown to be functional in *E. coli* crude extracts and showed affinity for UDP-D-galactose, similar to other UDP-galactose 4-epimerases. Further, the isolated *G. pristoides* UGE (*Gp*UGE) was biochemically characterized and its kinetic parameters determined. We found that there was no kinetic difference between this enzyme and previously described UGE enzymes except enhanced activity in the presence of exogenously added NAD^+.

The UDP-galactose 4-epimerase (UDP-glucose 4-epimerase, UGE, EC 5.1.3.2) is an essential Leloir pathway enzyme facilitating the catalytic inter-conversion between UDP-D-glucose and UDP-D-galactose. UDP-D-galactose is the nucleotide sugar required by galactosyltransferases for the production of red algae sulfated galactans. UGE is suspected as being responsible for supplying UDP-D-galactose for the synthesis of sulfated galactans. *In planta* monitoring of *Gp*UGE transcript levels with respect to dark and light cycling indicated high expression of the enzyme at night, while expression diminished during the day. The occurrence of increased nocturnal UGE expression correlates with floridean starch breakdown at night. Evidence for hydrolysis of floridean starch is also reflected in obtained *G. pristoides* transcriptome sequence data. In red algae, floridean starch degradation coincides with sulfated galactan production. The detection of starch hydrolysis enzyme transcripts alongside increased expression of *Gp*UGE suggests the enzyme plays a role in supplying UDP-D-galactose for sulfated galactan production. As far as we know, this the first report of sequencing and biochemical characterization of a UGE from red seaweed.

Aknowledgments

All glory goes to GOD for great things he has done. Thank you for giving me strength in my darkest hour and being a guiding light at the end of the tunnel. Without You none of this would have been possible.

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Dedication

To my late father Stephanus Jacobus Hector

To my daughters Luca and Annah, may education be your light

TABLE OF CONTENTS

CHAPTER 2

Bioprospecting for sulfated galactan biosynthesis genes from *Gelidium pristoides*

CHAPTER 3

Correlation of activity of the UDP-galactose 4-epimerase with galactan (agar) biosynthesis in Rhodophyte *Gelidium pristoides*

CHAPTER 4

De novo sequencing of *Gelidium pristoides* transcriptome

CHAPTER 5

General discussion

APPENDIXES

ABBREVIATIONS

Å	Ångström units
aa	Amino acids
Ap	Ampicillin
ATP	Adenosine-5'-triphosphate
Bp	Base pairs
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
cDNA	Complimentary DNA
Cm	Chloramphenicol
C-terminal	Carboxyl-terminus
ddd H_2O	Double distilled deionized water
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
EDTA	Ethylenediaminetetraacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
FPLC	Fast protein liquid chromatography
g	Gram
Galt	Galactose-1-phosphate uridylyltransferase

gDNA	Genomic DNA
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobase pairs or 1000-bp
k_{cat}	Turnover number
kDa	Kilodaltons
K_i	Inhibition constant
K_m	Michaelis constant
Km	Kanamycin
L	Liter
M	Molar
MES	2-(N-morpholino)-ethanesulfonic acid
ml	milliliters
mM	millimolar
MOPS	3-[N-Morpholino]-propanesulfonic acid
mRNA	messenger RNA
N	normal
N_2	Nitrogen
NAD^+	Nicotineamide adenine dinucleotide (oxidized)
NADH	Nicotineamide adenine dinucleotide (reduced)
ng	Nanograms
nt	Nucleotide
N-terminal	Amino-terminus
^oC	degrees Celcius

ORF	Open reading frame
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RBS	Ribosomal binding site
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
SDS	Sodium dodecyl sulfate
sqPCR	Semi-quantitative PCR
ssDNA	Single stranded DNA
Strep	Streptomycin
TE	Tris EDTA buffer
Tet	Tetracycline
UDP	Uridine diphosphate
UDP-gal	UDP-D-galactose
UDP-glc	UDP-D-glucose
UGD	UDP-D-glucose dehydrogenase
UGE	UDP-galactose 4-epimerase
UMP	Uridine monophosphate
µg	microgram
µl	microliter
v/v	volume/volume
V_{max}	Maximum rate
w/v	weight/volume
WT	wild type
X-Gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

CHAPTER 1

Sulfated galactans: An overview

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1.1. Polysaccharides

Condensed carbohydrates are the most abundant biomolecules on earth existing as oligo- or polysaccharides (Gomez d'Ayala *et al*., 2008; Ghazarian *et al*., 2011). These mostly linear molecules consist mainly of monomeric sugars (glucose, galactose, fructose etc.) linked via *O*-glycosidic bonds. Oligosaccharides are short chains composed of 10 sugar residues or less, while polysaccharides are typically longer. Heteropolysaccharides are linear arrangements composed of different types of sugar residues (glucose, galactose and mannose) whereas homopolysaccharides are more simplistic structures comprised of a single monomer group only i.e. either glucose, galactose, or fructose (Mathews and van Holde, 1996). Collectively, homo – and heteropolysaccharides play important roles as structural and storage molecules in plants and animals. Starch and glycogen, both polymers of α-1,4-linked glucose with α-1,6-linked branches are examples of homopolysaccharides used for storage (energy reserves) in bacteria and eukaryotes that can be readily metabolized to glucose for glycolysis (Martin and Smith, 1995; Shearer and Graham, 2002). Pectin is a heteropolysaccharide of branched α-1,4-D-galacturonic acid residues and contributes significantly to the integrity and rigidity of plant cell wall structure allowing for upright growth (Mohnen, 2008). Examples of homopolysaccharides providing structural capacity are cellulose, the most abundant polysaccharide in nature, and sulfated polysaccharides. In marine organisms, sulfated homopolymers play structurally a more significant role than cellulose (Percival, 1979). These polysaccharides occur in high concentrations in extracellular matrices performing important biological roles ranging from desiccation protection to fertilization (Percival, 1979; Pavão *et al*., 1989).

1.2. Structural diversity of marine sulfated polysaccharides

Sulfated homopolysaccharides are widely distributed amongst marine organisms including algae, echinoderms (sea cucumbers and urchins) and tunicates (ascidians). With the exception of mammalian glycosaminoglycans (GAGs) (i.e. heparin, chondroitin and dermatin), sulfated galactans and fucans are the most studied sulfated polysaccharides (Pomin and Mourão, 2008). Sulfated polymers from marine organisms are generally composed of (i) α-L-fucopyranosyl residues (fucans) or (ii) α-L-and/or α-D- or β-D-galactopyranosyl residues (galactans) (Pomin and Mourão, 2008; Pomin, 2009a and Pomin, 2009b). Structurally, this heterogeneous group of highly anionic compounds is well conserved amongst phyla, alluding to their diverse biological functionality.

1.3. Function and biological significance of sulfated galactans

Sulfated polysaccharides exist as either mucilages or gels in extracellular matrices of various marine species. Structural and biological roles include strengthening of cell walls, flexibility to resist wave action, desiccation prevention and maintenance of cellular ionic equilibrium (Percival, 1979). They have analogous function to glycosaminoglycans (GAGs) in terrestrial organisms, acting as structural cross-linking material (Albano *et al*., 1986; Pavão *et al*., 1989; Santos *et al*., 1992; Pomin and Mourão, 2008; Pomin, 2009a; Pomin, 2009b). Vertebrate GAGs are not as extensively sulfated as galactans and fucans. This high degree of sulfate substitutions are advantageous in saline environments as they coincide with high electro-negative charge densities promoting enhanced interaction between extracellular matrix constituents in marine organisms (Pomin and Mourão, 2008).

In addition to being a structural bonding material, sulfated galactans also fulfill biological roles. The non-covalently linked sulfated galactans located in the peripheral jelly layer surrounding sea urchin eggs facilitate gamete recognition necessary for sea urchin egg fertilization (Alves *et al*., 1997; Vilela-Silva *et al*., 2008; Pomin, 2009b; Castro *et al*., 2009). During fertilization it is involved in carbohydrate-induced signal transduction leading to the Acrosome reaction, a series of physiological events triggered by sperm and sulfated galactan interactions, causing egg cell adherence (Figure 1.1.). Sea urchin sperm-egg recognition

mechanisms are strictly species specific and therefore demonstrate the intimate relationship between sulfated polysaccharides structural features and their biological function (Pomin, 2009)

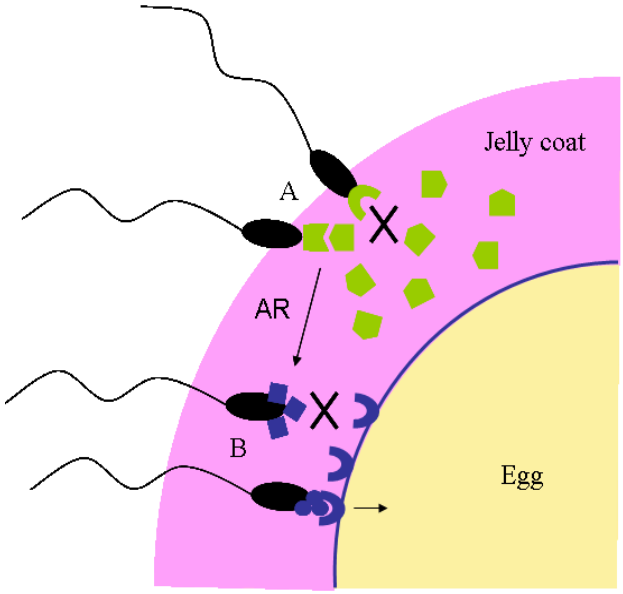

Figure 1.1. Schematic representation of sea urchin gamete recognition (adapted from Pomin and Mourão, 2008). (**A**) Species specific sulfated galactan-based gamete recognition. The Acrosome reaction (AR) is induced when the sperm head receptor recognizes the specific sulfated galactan in the jelly coat. (**B**) The protein binding coating resulting from the AR in turn leads to a reaction with the matching egg membrane receptor. Sperm receptor and galactan incompatibility fails to induce AR.

1.3.1. Sulfated galactans

The most abundant marine polysaccharides are the sulfated galactans (Fredericq *et al*., 1996). Distributed widely amongst marine organisms, these high molecular weight compounds are primarily localized in the cell walls of red and green seaweed, marine invertebrates (ascidians and sea urchins) and some sea grass species. Despite their broad phylogenetic distribution, the sulfated galactans all share relatively similar galactose backbone structures, but differ highly with respect to substituent sulfate groups (Figure 1.2.).

1.3.1.1. Red algae sulfated galactans

Sulfated galactans from red seaweed are classified into two distinct groups namely, agarans and carrageenans (Figure 1.2.A). Stereochemistry suggests that agarans consist mainly of alternately arranged 4-linked α-L-galactopyranose and 3-linked β-D-galactopyranose linear repeating disaccharide units (Araki, 1966). Carrageenans consist mainly of 4-linked α-L-galactopyranose and 4-linked α-D-galactopyranose (Usov, 1998). Apart from the galactose backbone, red algal galactans contain considerable portions of 3,6-anhydro-galactose residues and varying degrees of methyl, pyruvic acid acetyl and xylosyl groups. The sulfation pattern on the galactose backbone is thus a major contributor to structural heterogeneity.

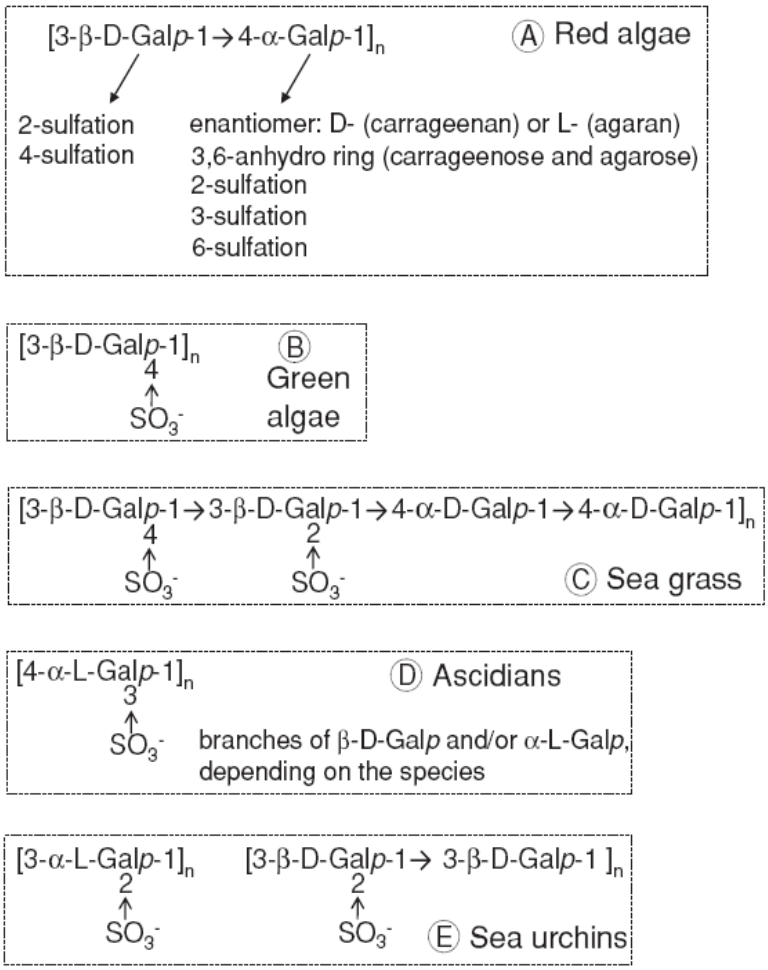

Figure 1.2. Linear representations of the repetitive units of diverse sulfated galactans (adapted from Pomin and Mourão, 2008). (**A**) Red algae (Usov, 1998; Lahaye ,2001, Pereira *et al*., 2005; Pomin and Mourão, 2008) (**B**) Green algae (Bilan *et al*., 2007; Farias *et al*., 2008) (**C**) Sea grass (Aquino *et al*., 2005) (**D**) Ascidians (Tunicates) (Mourão and Perlin, 1987; Pavão *et al*., 1989; Santos *et al*., 1992) (**E**) Sea urchins (Alves *et al*., 1997).

1.3.1.2. Sulfated galactans from invertebrates

Polysaccharides derived from marine invertebrates are generally more homogenously composed of repeating galactose units compared to those from marine algae (red and green). Sulfated galactans were first described in ascidians (tunicates or sea squirts) (Mourão and Perlin, 1987; Pavão *et al*., 1989) and later in enchinoderms (sea urchins) (Alves *et al*., 1997). The galactan isolated from the tunic of the ascidian *Styela plicata* is composed of 3-sulfated, 4-linked α-L-galactopyranose residues (Mourão and Perlin, 1987). Sea urchin galactans isolated from *Echinometra lucunter* (Alves *et al*., 1997) and *Glyptocidaris crenularis* (Pomin and Maurão, 2008) consist of 2- sulfated, 3-linked galactose units, which are more structurally related to green algal galactans (Figure 1.3.).

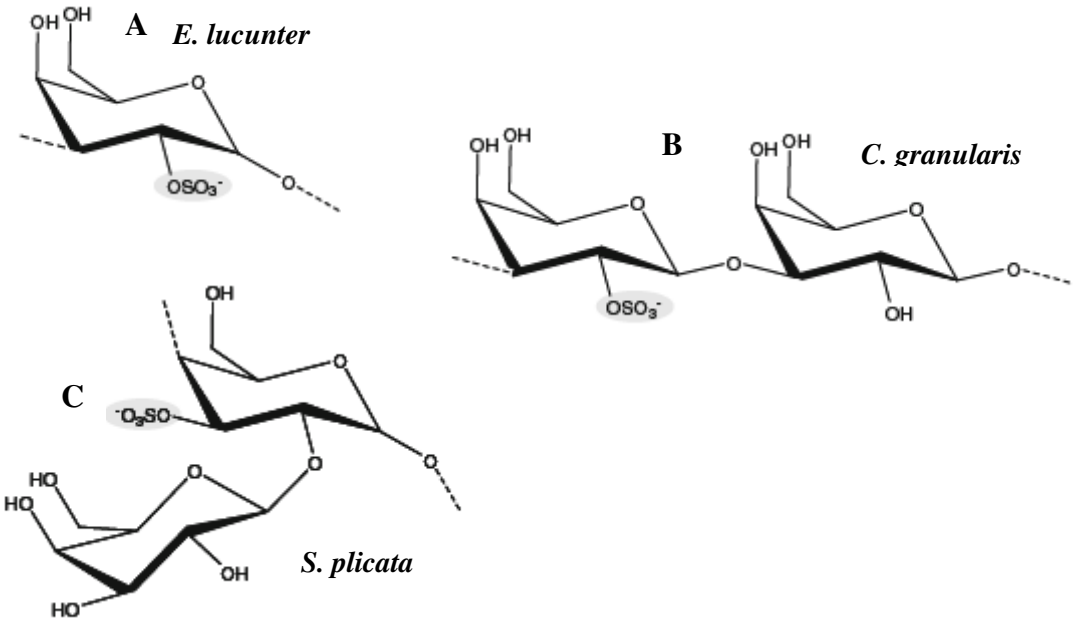

Figure 1.3. Structural comparison of the sulfated galactan (SG) repeating units of marine invertebrates (adapted from Pomin, 2009b). (**A**) SG from sea urchin egg jelly of *Echinometra lucunter* [→3-α-L-Galp-2(OSO_3^-)-1→]n (**B**) SG from *Glyptocidaris crenularis* [→3-α-L-Galp-2(OSO_3^-)-1→3-α-L-Galp-1→]n (**C**) SG from Ascidian tunic *Styela plicata* {→4)-α-LGalp-2[→1)- α-L-Galp]-3(OSO_3^-)-(1→}n. Sulfate substitution groups (OSO_3^-) are highlighted in grey on structure representations.

1.3.1.3. Occurrence of sulfated galactans in marine angiosperms

More recently, sulfated galactans have been described in marine angiosperms (Aquino *et al*., 2005). A D-galactan isolated from the seagrass *Ruppia maritima* was shown to be composed of tetrasaccharide repeating units of both α- and β-D-galactose (Figure 1.4.E.), similar to red algal galactans. The galactose residues, however, are not arranged in alternating order as those found in red algae. Instead they consist of a 4-sulfated, 3-linked β-D-galactopyranose backbone structures (Aquino *et al*., 2005; Farias *et al*., 2008). However, polymers described from related species *Codium isthmocladum* contained 6-sulfated and 6-linked β-D-galactose monomers (in small amounts) (Farias *et al*., 2008). Of all reported sulfated galactans from marine fauna and flora, the green algal galactans display most structural complexity (Figure 1.4.).

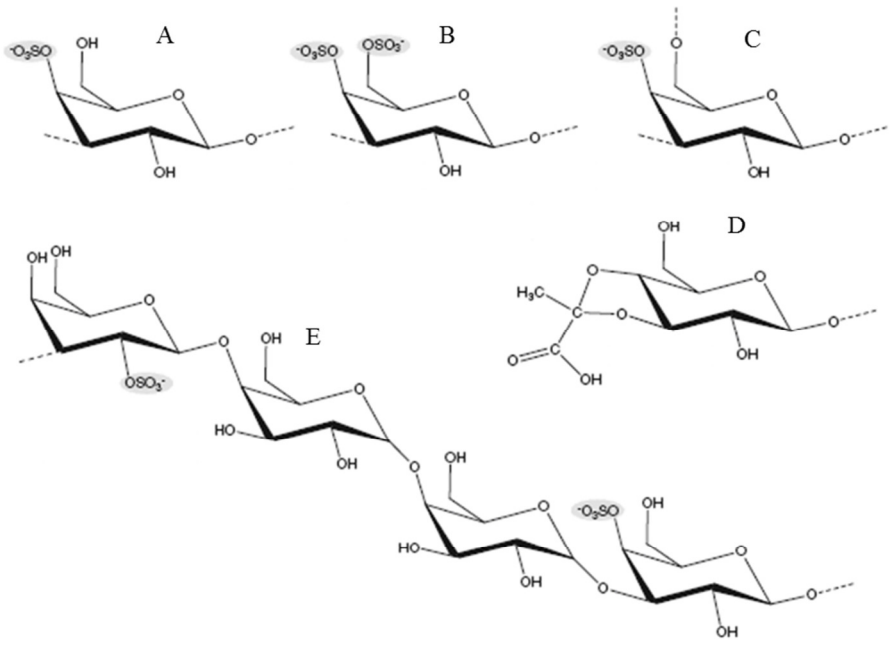

Figure 1.4. Structural comparison of sulfated galactans (SG) from green algae and marine angiosperms (adapted from Pomin 2009b). Green algal SG isolated from *Codium isthmocladum*: (**A**) 3-β-D-galp-4(OSO_3^-) with minor constituents, (**B**) 3-β-D-galp-4,6-di(OSO_3^-), (**C**) 6-β-D-galp-4(OSO_3^-), and (**D**) 3,4-O-(1-carboxy)-ethylidene-β-D-galp-1. (**E**) SG from the marine angiosperm *Ruppia maritima*: [→3-β-D-galp-2(OSO_3^-)-1→4-α-D-galp-1→4-α-D-galp-1→3-β-D-galp-4(OSO_3^-). Sulfate substitution groups (OSO_3^-) at various positions are highlighted in grey in sketch.

1.4. Sulfated galactans (agarans and carrageenans) occurring in red macro algae (Rhodophyta)

Sulfated galactans are of great industrial importance and have valuable application in food, cosmetic and pharmaceutical industries as a result of their gelling and stabilizing abilities (De Ruiter and Rudolph, 1997; Tseng, 2001). These polysaccharides usually have linear backbones of alternating 3-linked β-D-galactopyranose and 4-linked α-L-galactopyranose or 4-linked α-D-galactopyranose units, displaying "masked repeating" chains of dissacharide residues (Anderson and Rees, 1966). Porphyran, obtained from *Porphyra spp*., is one of the best studied agarans and has archetypal agaran structure of alternating 3-linked β-D-galactopyranose and 4-linked α-L-galactopyranose-sulfate (Morrice *et al*., 1984). The presence of 4-linked 3,6-anhydro galactose derivatives, in conjunction with sulfation patterns are used for galactan classification (Craigie, 1990; Knutsen *et al*., 1994; van de Velde *et al*., 2004).

Pioneering studies successfully separated agar into two fractions (i) neutral agarose and (ii) anionic porphyran (agar) (Araki, 1966; Araki *et al*. 1967). Today, an abundance of agar-related polysaccharides have been described and the generic term 'agar' used to describe all gel-forming polymers is no longer applicable. Classification and nomenclature systems related to sulfated galactans have been revised several times (Craigie, 1990; Knutsen *et al*., 1994; Usov, 1998; Lahaye, 2001). The system proposed by Knutsen and co-workers deserves mention as it satisfies both chemical (IUPAC) and logical desire. They referred to agarose as the "ideal" structure since it consists almost entirely of α-1-4-linked 3,6-anhydro-L-galactose residues. In contrast, the term agaran was used for polymers deviating from this "ideal" state i.e. compounds with alternating 3-linked β-D-galactopyranose and 4-linked α-L-galactopyranose residues containing various substituent groups.

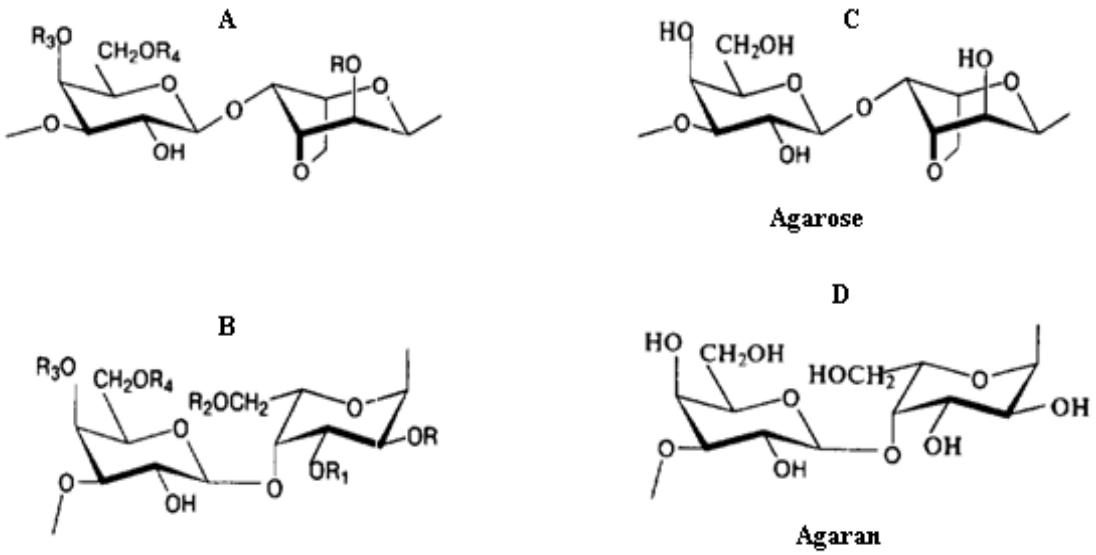

Figure 1.5. Chemical structures of agar related polysaccharides (adapted from Usov, 1998). (**A**) and (**B**) Example of agarans with substituent groups: R = H or Me; R_1 = H, Me or β-D-Xylose; R_2 = H or SO_3^-; R_3 = H or SO_3^-; R_4 = H, Me, SO_3^-, β-D-Xylose or 4-OMe-α-L-Galactose. (**C**) and (**D**) Agarose and agaran as described by Knutsen et al. (1994).

Previously, Greek prefixes were assigned to carrageenans based on their gelling ability in the presence of certain ions. For example, kappa-carrageenan (κ-carrageenan) and iota carrageenan (ι-carrageenan) precipitate in the presence of potassium and calcium ions, respectively (Lahaye, 2001; Campo *et al*., 2009). More recently, nomenclature procedure depends on specific groups representing different classes of sulfate substitution (Craigie, 1990; Knutsen *et al*., 1994; Lahaye, 2001). Accordingly, they were divided into 3 families i.e. (i) kappa, (ii) beta and (iii) lambda carrageenans based on their sulfation patterns on specific galactose residues. The short hand nomenclature system proposed by Knutsen et al. (1994) was adopted for carrageenan classification (Table 1.1) (Lahaye, 2001; van de Velde *et al*., 2001, De Ruiter and Rudolph, 1997; Campo *et al*., 2009).

***Table 1.1.** Short hand symbols developed by Knutsen et al. (1994) for agar and carrageenan nomenclature.

Letter code	Corresponding name
D	4-linked α-D-galactopyranosyl
DA	4-linked 3,6-anhydro-α-D-galactopyranosyl
G	3-linked β-D-galactopyranosyl
L	4-linked α-L-galactopyranosyl
LA	4-linked 3,6-anhydro-α-L-galactopyranosyl
M	*O*-methyl
P	4,6-*O*-(1-carboxyethylidene)
S	Sulphate ester
L2M,6S[b]	4-linked 2-*O*-methyl-α-L-galactopyranosyl 6-sulphate
-G4S-DA-G-	->3) β-D-galactopyranosyl 4-sulphate (1->4) 3,6-anhydro-α-D-galactopyranosyl (1->3) β-D-galactopyranosyl (1->

*Lahaye (2001).

The terms carrageenan is restricted to alternating 3-linked β-D and 4-linked α-D galactans, while carrageenose refers to "ideal" (4-linked 3,6-anhydro-α-D-galactose) polysaccharides (Craigie, 1990; Knutsen *et al.*, 1994; De Ruiter and Rudolph, 1997; Usov, 1998). The systematic naming of carrageenan and agaran-related polysaccharides coincided simultaneously with development of shorthand letter codes (Table 1.1.). For example 4-Linked D- and L-galactose units are represented by the letters D and L, DA and LA refer to 4-linked D- and L-anhydro derivatives, respectively. On the other hand, the letter G refers to 3-linked β-D-galactose residues. Shorthand symbols were also developed for substituent groups denoting their carbon positions on respective galactose residues (Figure 1.5.) (M being for methyl ether, S for sulfate ester and P for pyruvate). "Ideal" kappa-carrageenan (carrageenose 4-sulfate) is G4S-DA and "ideal" iota-carrageenan (carrageenose 2,4-disulfide) is G4S-DA2S. Agarose would be G-LA, and mu- and nu-carrageenan represented as G4S-D2S,6S and G4S-D6S, respectively (Figure 1.6.) (De Ruiter and Rudolph, 1997; Miller, 1997; Lahaye, 2001; van de Velde and De Ruiter, 2002, van de Velde *et al.*, 2004).

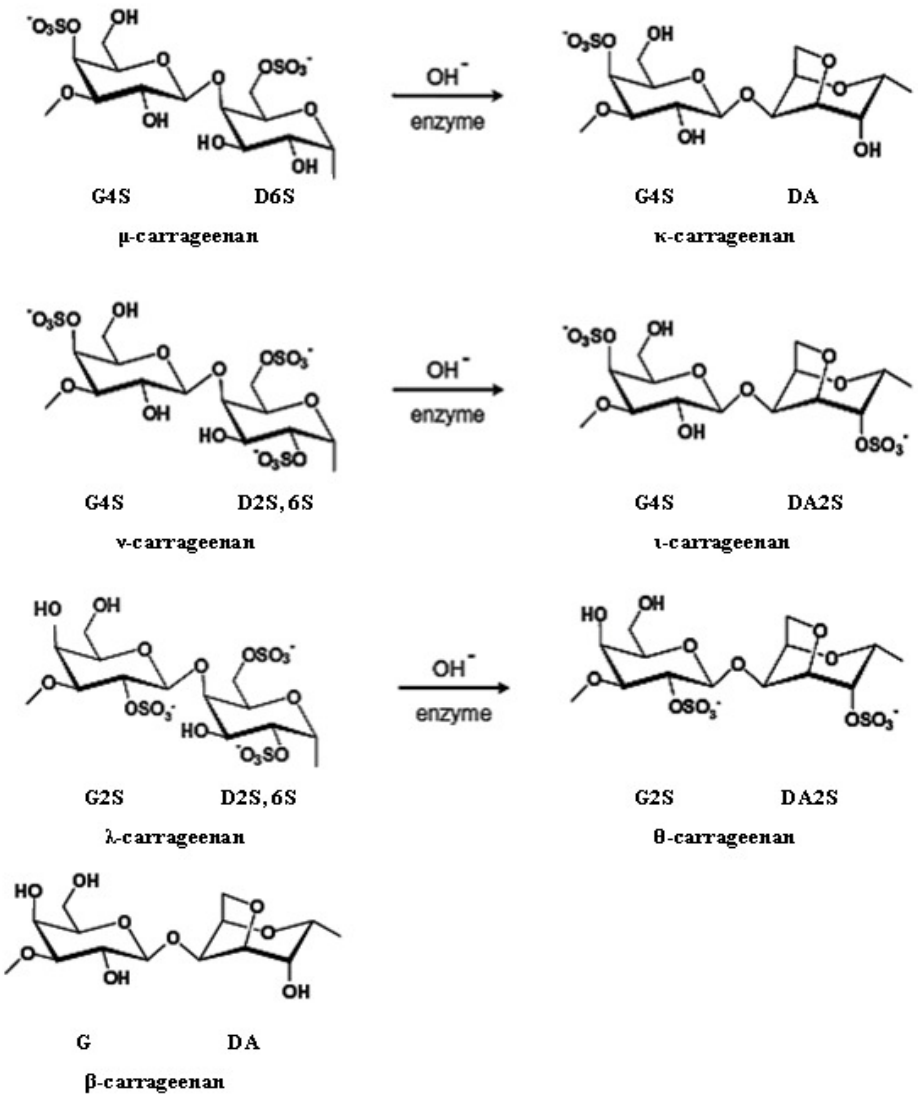

Figure 1.6. Different carrageenan repeating disaccharides indicated using shorthand notation proposed by Knutsen *et al*., (1994).

1.5. Biosynthesis of sulfated galactans

In contrast to genetic understanding of sulfated galactan production, the chemistry of sulfated galactans is fairly well studied. The current hypothetical model is based on a combination of terrestrial plant (*Viridiplantae*) and red algal (*Rhodophyta*) biochemistry. Biosynthesis of sulfated galactans is thought to occur in three sequential steps involving three main classes of enzymes (Rees 1961a and 1961b; Hemmingson *et al*., 1996a; Genicot-Joncour *et al*., 2009). During the first step, galactosyltransferases catalyze chain elongation via the transfer of

activated galactose residues. Secondly, sulfotransferase enzymes decorate the galactose backbone with sulfate ester groups. Finally, galactose-6-sulfurylases or sulfohydrolases are responsible for conversion of galactose-sulfate moieties to 3,6-anhydro ring residues.

Inferred from red algae biochemistry, two classes of enzymes i.e. α-1,4 and β-1,3-galactosyltransferases mediate synthesis (known as galactosylation) of the galactan backbone requiring GDP-L-galactose and UDP-D-galactose, respectively. These galactose enantiomers are derived from D-glucose and D-mannose supplied by different biosynthesis pathways. The synthesis of GDP-L-galactose (the substrate for α-1,4-galactosyltransferases) is a product of the D-mannose/L-galactose pathway (Major *et al*., 2005). Production of GDP-L-galactose is catalyzed by the enzyme GDP-mannose 3',5'-epimerase (GME, EC 5.1.3.18). GME catalyzes twin step epimerization reactions at positions C3′ and C5′ of GDP-D-mannose yielding the nucleotide sugar GDP-L-galactose (Wheeler *et al*., 1998; Major *et al*., 2005). On the other hand, UDP-D-galactose, the substrate for β-1,3-galactosyltransferases, is synthesized by the Leloir pathway (Leloir, 1951). It can be synthesized by either galactose-1-phosphate uridylyltransferase (GALT, EC 2.7.7.12) which transfers UDP from UDP-D-glucose to galactose-1-phosphate generating UDP-D-galactose, or by UDP-galactose 4-epimerase (also known as UDP-glucose 4-epimerase, GALE, UGE, GAL10, EC 5.1.3.2) by conversion of UDP-D-glucose to UDP-D-galactose (Figure 1.10.) (Frey, 1996). Both enzymes form part of the Leloir pathway and have been extensively studied in prokaryotes and eukaryotes (Dai *et al*., 2006).

In algae and terrestrial plants, nucleotide sugars including GDP-L-galactose and UDP-D-galactose are synthesized in the cytosol (Goulard *et al*., 1999). These activated sugars are relocated to the Golgi lumen by specific nucleotide sugar transporters (NSTs) embedded in the Golgi membrane (Gibeaut, 2000; Handford *et al*., 2004). NSTs function as anti-porter systems actively importing nucleotide sugars into the lumen and exporting nucleotide monophosphates (NMP). Thus far, plant NSTs has been identified in pea (*Pisum sativum*), rice (*Oryza sativa*) and *A. thaliana* (Munoz *et al*., 1996; Handford *et al*., 2004; Zhang *et al*., 2010). Interestingly, in rice it has been shown that NSTs modulate cell wall biosynthesis (Zhang *et al*., 2010). Similar to NSTs, galactosyltransferases (and glycosyltransferases in general) are also Golgi-located (Colley 1997; Hennet 2002). At the Golgi site, galactosyltransferases are in close proximity to imported nucleotide sugars which can readily

be assembled into matrix polysaccharides (Gibeaut, 2000; Keegstra and Raikhel, 2001; Breton *et al*., 2006). Although, molecular information on red algae galactosyltransferases is unknown, bacterial and terrestrial plant homologues have been well studied and reviewed (Keegstra and Raikhel, 2001; Hennet, 2002; Berger and Rohrer, 2003). Thus, their mechanisms provide models for sulfated galactan-associated galactosyltransferases. Polymerization mechanisms of nucleotide sugars by galactosyltransferases can be labeled as retaining or inverting depending on the configuration of the anomeric carbon during transfer from nucleotide sugar to acceptor substrate (Campbell *et al*., 1997; Gibeaut, 2000; Breton *et al*., 2006).

Sulfated galactan heterogeneity depends largely on sulfotransferases (Pomin and Mourão, 2008). Based on structural studies sulfation is known to occur at four different positions (C-2, C-3, C-4 and C-6). These positions are organism specific and each requires different sulfotransferases. Current knowledge suggests Golgi-localization of these trans-membrane enzymes (Tveter-Gallagher *et al*., 1984; Gretz and Wu, 1990, Strott, 2002, de Graffenried *et al*., 2004). Although molecular data on red seaweed sulfotransferases remain elusive, an EST showing sulfotransferase activity was recently described for red micro algae (Arad and Ontman, 2010). Moreover, further detail surrounding biochemical characterization of this enzyme remains elusive. Reaction mechanisms for bacterial and human sulfotransferases are well described (Mougous *et al*., 2002; Strott, 2002; Gamage *et al*., 2006). These enzymes are similar in action and all require PAPS (3'-phosphoadenosine 5'-phosphosulfate) as sulfate donor. It is generally accepted that a synthase complex facilitates transfer of sulfate (from PAPS) to acceptor substrates (for e.g. polysaccharides, proteins, vitamins and steroids) subsequently releasing of desulfated PAP (Figure 1.7). Although the overall catalytic mechanism has been elucidated it is not yet known whether neutral galactans are sulfated or individual galactose moieties are decorated after chain elongation. It is, however, suspected that residues are sulfated prior polymerization and export (Cardozo *et al*., 2007).

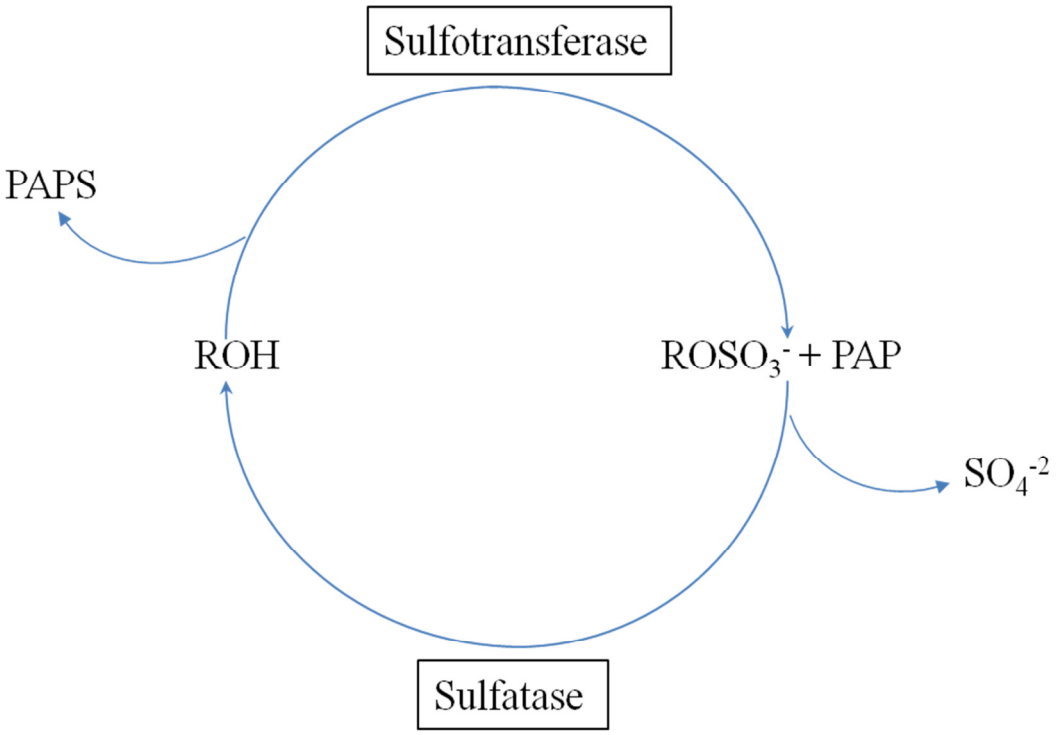

Figure 1.7. General proposed mechanism of sulfation of polysaccharides in bacteria and humans (Strott, 2002).

The final step in galactan biosynthesis is 3,6-anhydro-galactose ring closure (Hemmingson *et al*., 1996a). The formation of 3,6-anhydro linkages are sterically important allowing for superior helix formation in aqueous environments. Consequently, abundant sulfate ester groups are associated with poor gelling ability (Tuvikene *et al*., 2007). Apparently, sulfate groups introduce kinks in the overall structure promoting unfavorable water interaction rendering a poorer quality product. Pioneering studies performed by Rees (1961a and 1961b) correlated the release of sulfate from the galactan porphyran with the formation of 3,6-anhydro-galactose moieties in the polysaccharide chain. Later, similar results were demonstrated by pulse fed radio-labeling *in planta* experiments in red algae (*Gracilaria chilensis*) (Hemmingson *et al*., 1996a). These authors suggested that a decrease in galactan sulfate groups correlated with increased 3,6-anhydro-galactose content. Recently, Genicot-Joncour and colleagues (2009) isolated two enzymes (Sulfurylase 2 and 6) from *Chondrus crispis* with the ability to catalyze anhydro-bridge formation (Genicot-Joncour *et al*., 2009).

These enzymes catalyze conversion of nu- (ν-) to iota (ι-) carrageenan via formation of 3,6-anhydro-galactose releasing free sulfate in the process. The precise enzymatic mechanism of the two enzymes remains unknown and requires further study. The authors proposed nucleophilic substitution as the most likely mode of action.

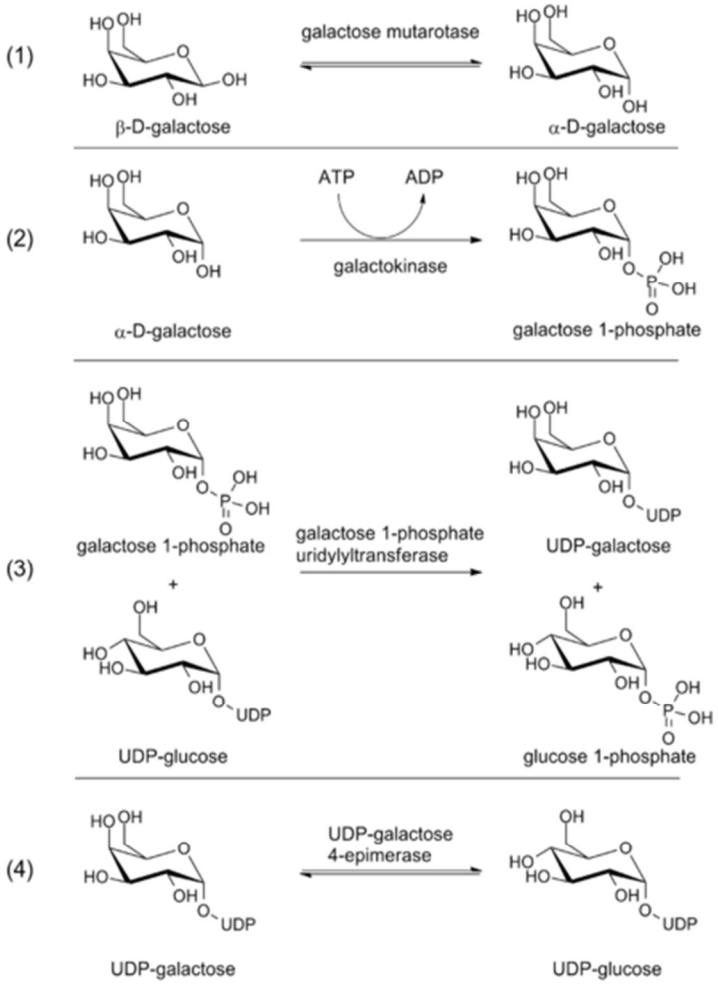

Figure 1.8. Step by step enzymatic production of galactose through the Leloir pathway. The pathway is dedicated to the sequential reversible enzymatic conversion of UDP-D-glucose to UPD-galactose (Leloir 1951). The putative Leloir enzymes are as follows: (1) galactose mutarotase (EC 5.1.3.3) (2) galactokinase (EC 2.7.1.6) (3) galactose-1-phosphate uridylyltransferase (EC 2.7.7.12) (4) UDP-galactose 4-epimerase (EC 5.1.3.2).

1.7. Precursors of sulfated galactan biosynthesis

More experimental evidence is needed in order to elucidate mechanisms surrounding sulfated galactan biosynthesis. However, evidence preceding biosynthesis are substantial and in conjunction with hypothetical models may provide an overall perspective of biosynthesis. Present evidence suggests initial biosynthesis steps occur from an existing carbohydrate pool. The main photosynthetic product of macro red algae is floridean starch, which serves as primary carbon sink for sulfated galactan biosynthesis (Rotem *et al*., 1986; Craigie, 1990; Hemmingson *et al*., 1996a; Collén *et al*., 2004). Similar to starch in terrestrial plants, floridean starch is accumulated during periods of irradiance and is metabolized in the absence of light (Collén *et al*., 2004). Floridean starch is degraded during respiration to form UDP-D-glucose via UDP-D-glucose pyrophosphorylase (Figure 1.8.) (Lluisma and Ragan, 1999; Goulard *et al*., 2001). The Leloir enzyme, UDP-galactose 4-epimerase, subsequently interconverts UDP-D-glucose to UDP-D-galactose (Figure 1.8.) (Frey, 1996). Various studies (in terrestrial plants and algae) support its involvement in cell wall polysaccharide synthesis as supplier of UDP-D-galactose (Joersbo *et al*., 1999; Seifert *et al*., 2002; Barber *et al*., 2005; Rösti *et al*., 2007). In red algae UDP-D-galactose was shown to be the most abundant nucleotide sugar (Manley and Burns, 1991; Goulard *et al*., 1999). As producer of UDP-D-galactose, UDP-galactose 4-epimerase is an important precursor enzyme for sulfated galactan production (Prosselkov *et al*., 1996; Fournet *et al*., 1999; Goulard *et al*., 1999; Goulard *et al*., 2001; Goulard *et al*., 2003). It has been cloned from various organisms, however it has only been chromatographically purified from some red algae species (*Galdieria sulphuraria* and *Solieria chordalis*) (Prosselkov *et al*., 1996; Goulard *et al*., 2003). Over the years, the enzyme has attracted significant research attention because of its interesting catalytic mechanism (See Chapter 3 introduction) (Thoden and Holden, 1996; Barat and Bhattacharyya, 2001; Holden *et al*., 2003; Brahma and Bhattacharyya, 2004; Nayar *et al*., 2004; Thoden and Holden, 2005, Brahma *et al*., 2009; Kim *et al*., 2011). However, studies regarding its expression in relation to sulfated galactan synthesis have not been considered.

GDP-D-mannose alongside UDP-D-glucose forms part of the general carbohydrate pool in plants and algae (Manley and Burns, 1991; Reiter and Vanzin, 2001). It is synthesized by GDP-D-mannose pyrophosphorylase from GTP and mannose-1-phosphate (Feingold and Avigad, 1980). Besides serving as substrate for the biosynthesis of mannose-containing cell wall polysaccharides, the nucleotide sugar is also required for the synthesis of GDP-L-galactose (Figure 1.9.). GDP-L-galactose is an important nucleotide sugar which acts as the L-galactose donor for biosynthesis of sulfated galactans (Reiter and Vanzin, 2001). In nature

L-galactose (a derative of GDP-L-galactose) is relatively rare (Tang, 2012). However, its abundance in marine sulfated galactans (asidians and algae) illustrates the importance of GDP-L-galactose for sulfated galactan biosynthesis. In algae (and higher plants) GDP-L-galactose is synthesized by the enzyme GDP-mannose 3',5'-epimerase (GME; E.C. 5.1.3.18) via dual-coupled epimerization of C-3' and C-5' of GDP-mannose. The enzyme is well described for terrestrial plants as part of the Wheeler-Smirnoff pathway for L-ascorbic acid biosynthesis (Wheeler *et al*., 1998; Wheeler and Smirnoff, 2000; Wolucka *et al*., 2001; Wolucka and Montagu, 2003; Major *et al*., 2005). Recently, a GME gene was characterized from red algae *Gracilaria changii* (Siow *et al*., 2013). The enzyme was shown to produce GDP-L-galactose and real-time monitoring of GME expression was linked to increased agar production. At present, evidence suggests GME (and UGE) are essential precursor enzymes in red algae and participate in conjunction with galactan synthases in sulfated galactan production.

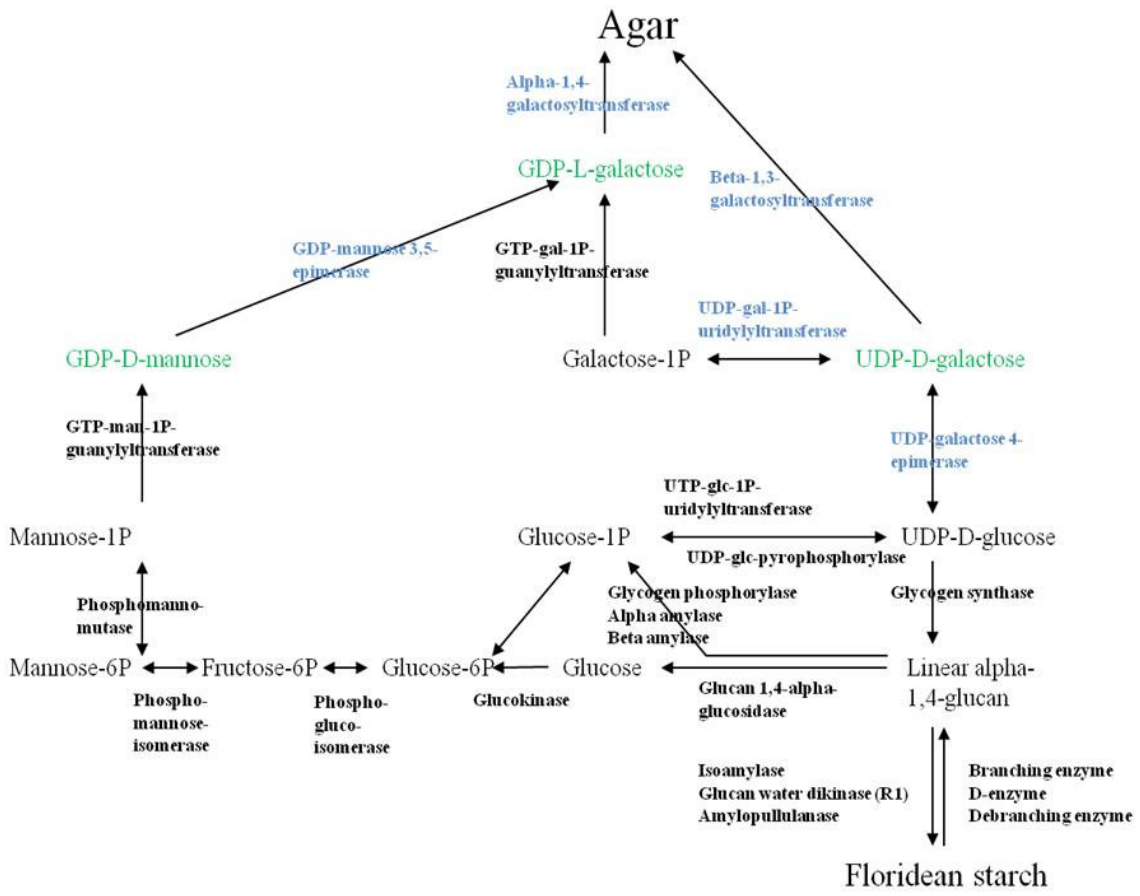

Figure 1.9. The proposed pathway for agar biosynthesis in red algae (adapted from Barbier *et al.*, 2005). Floridean starch that is accumulated during daylight hour is subsequently degraded to linear glucans and glucose. Glucose is further converted via two different pathways to glucose-1-phosphate and glucose-6-phosphate, respectively. These metabolites through enzymatic conversion become precursor sugars UDP-glc and GDP-man. The nucleotide sugars in turn are epimerized (via GDP-mannose 3,5-epimerase and UDP-galactose 4-epimerase) to GDP-L-galactose and UDP-D-galactose prior to export to the Golgi apparatus for incorporation into polysaccharides by α-1,4 and β-1,3-galactosyltransferases.

1.7. Industrial applications of sulfated galactans

1.7.1. Sulfated galactans as potential pharmaceutical therapeutics

Research interest in structural and functional biology surrounding sulfated polysaccharides from algae (Rhodophyta, Phaeophyta and Chlorophyta) was initiated in the late 80's (Mourão and Perlin 1987; Mourão 1990). This was partially due to advancement in analytical techniques for e.g. nuclear magnetic resonance (NMR) and mass spectrometry (MS), which could be applied for analysis of these unique polymers. During this early period it was

discovered that the biological activities of these high molecular weight compounds possessed great pharmacological and medicinal value. Today, research focus is on a wide range of sulfated polysaccharides for pharmaceutical application i.e. anti-viral, anti-thrombotic, anti-coagulation and anti-inflammatory activities (Pomin, 2012).

1.7.1.1. Anti-viral potency

Sulfated galactans have shown extensive anti-viral activity towards herpes simplex (HSV), respiratory syncytial virus (RSV) and dengue virus infections (Carlucci *et al*., 1997; Carlucci *et al*., 1999; Talarico *et al*., 2005, Matsuhiro *et al*., 2005). These polysaccharides show activity during the initial stages of cell surface adsorption exhibited by some RNA-based viruses (De Clercq, 1996; 2000). Carrageenans from *Gigartina skottsbergii* and a galactan from *Schizymenia binderi* deserve mention due to potent inhibitory effects against *Herpes simplex* virus (HSV) types 1 and 2 possibly preventing viral adsorption onto host cells (Carlucci *et al*., 1997; Talarico *et al*., 2005, Matsuhiro *et al*., 2005). Topical treatments derived from these galactans have since been developed for protection against vaginal HSV-2 and murine infection (Talarico *et al*., 2004).

1.7.1.2. Anti-thrombotic and anticoagulant agents

The most significant advancements in sulfated polysaccharide research have been geared toward anti-thrombotic and anticoagulant applications as alternatives to heparin (Mourão and Pereira, 1999). Heparin has been used for anti-thrombotic therapy for more than 50 years. Administration of this compound is inherently problematic as it stimulates allergic responses in certain patients and some hemorrhagic side effects have also been reported. Heparin administration also raises ethical concerns as most preparations are derived from animal sources (Pomin, 2012). These findings motivate investigations to find potential replacements for the therapeutic. It is well known that galactan structural heterogeneity arises from sulfated galactoseresidues (Lahaye, 2001; Campo *et al*., 2009). This feature also significantly contributes to its pharmacological action. Consequently, all sulfated polysaccharides exhibit anti-thrombin and anti-coagulant properties, although some are more potent than others (Farias *et al*., 2000; Pereira *et al*., 2005). In particular, the anticoagulant potency of a 2,3-di-sulfated galactan produced by *Botryocladia occidentalis* (Rhodophyta) is a noteworthy example (Pereira *et al*., 2005). Potency was attributed to extensive sulfation which directly

contributes to interaction with coagulant stimulators (Fonseca *et al*., 2008). These authors noted that the 2,3-di-sulfated D-galactan displays therapeutic activities comparable to heparin, highlighting its pharmacological potential as an alternative treatment for venous thrombosis.

1.7.2. Essential reagents for molecular biology applications

Perhaps the most widely recognized and lucrative application for sulfated galactans is in the field of molecular biology. Agarans are of high commercial value due to its utilization as solidifying agent in solid growth media for cultivation of microorganisms. Agarose, a neutral galactose polysaccharide derived from processed agar, is the key component of electrophoresis gels allowing separation and visualization of DNA molecules (Radmer, 1996). Cumulatively, recorded annual sales for agar and agarose were $210 million in the 90's (Radmer, 1996).

1.7.3. Food application

Seaweeds have been utilized as food source by East Asians for hundreds of years (Tseng, 2001) and health benefits associated with consumption are well known. China relinquished traditional algae farming methods in 1950 in favor of biotechnological approaches. Presently, they have 6 aquaculture industries dedicated to cultivation of commercially important species of which 4 are dedicated to algae used for consumption (Tseng, 2001). Sulfated galactans (carrageenans and agarans) extracted from red algae are used as thickeners, stabilizers and gelling of food (e.g. yoghurt) due to unique physico-chemical properties (Delattre *et al*., 2011).

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1.9. AIMS OF THIS STUDY

Worldwide red seaweed is commercially exploited for production of sulfated galactans (Radmer, 1996; De Ruiter and Rudolph, 1997). Red seaweeds are still mostly harvested manually from natural populations using traditional methods. Similarly, South Africa red seaweed species are not exempt from conventional harvesting methods. Traditional methods were found to have negative ecological effects on wild populations of algae and intertidal fauna (Anderson *et al*., 1990). In an attempt to conserve wild South African red seaweed populations, we aim to isolate the genetic determinants responsible for biosynthesis of galactan polymer production in red algae for expression in more suitable hosts (e.g. microorganisms or sugar cane). Expression of these genes in heterologous hosts would allow for large scale production of these polysaccharides circumventing harvesting of wild algae. It is widely accepted that galactosyltransferases are responsible for sulfated galactan propagation in red algae (Pomin and Mourão, 2008; Genicot-Joncour *et al*., 2009). However, to date, their gene sequences have not been elucidated.

Objectives of this study are summarized below:

- Identification of a suitable agar-producing red algae, which could be readily sampled from the South African coastline
- Construction of expression cDNA libraries from mRNA extracted from a South African agar-producing red algae species (*Gelidium pristoides*)
- Generation of a *S. cerevisiae* Gal10 i.e. UDP-galactose 4-epimerase mutant for functional screening of cDNA libraries.
- Introduction of an *Arabidopsis thaliana* GDP-mannose 3,5-epimerase gene into *S. cerevisiae* Gal10 to enable production of GDP-L-galactose.
- Identification of galactosyltransferase genes involved in agar biosynthesis through functional complementation of generated mutants
- Complementation of *S. cerevisiae* and *E. coli* mutants with library isolated endogenous *G. pristoides* UGE genes was expected. Thus far, red algae UGE homologues have remained largely unidentified. Although, previous reports have suggested UGE involvement in sulfated galactan production, their function have not yet fully been investigated in relation to production of these polysaccharides.

CHAPTER 2

Bioprospecting for sulfated galactan biosynthesis genes from *Gelidium pristoides*

2.1. INTRODUCTION

Glycosyltransferases (GTs) are a large family of enzymes governing the biosynthesis of numerous oligosaccharides and polysaccharides. Carbohydrates such as starch, glycogen, glycoconjugates and cell wall polysaccharides may function as biological storage molecules, structural scaffolds and signaling molecules. GTs catalyzes the transfer of the galactose moiety of activated nucleotide sugars to acceptor substrates to form glysodically-linked polysaccharides (Coutinho *et al*., 2003; Breton *et al*., 2006; Hashimoto *et al*., 2009). These enzymes are classified into 94 carbohydrate active enzyme (CAZY, www.cazy.org) families based on glycosidic linkages, reaction mechanisms (inverse or retaining actions), structural folds (i.e. GT-A or GT-B) and substrate specificities (Coutinho *et al*., 2003; Cantarel *et al*., 2008; Lairson *et al*., 2008). Galactosyltransferases (GalTs), a sub-family of the glycosyltransferases, contribute significantly to glycosylation processes in living tissues through the transfer of galactose (in α- and β- configurations) to form structural and cell wall polysaccharides (Hennet, 2002). These Golgi-localized enzymes are ubiquitously expressed in various tissue types and have widespread activity in living organisms. Despite low homologies, one component of catalytic reactions is identical; the use of UDP-D-galactose as a substrate for galactosylation reactions (Hennet, 2002; Berger and Rohrer, 2003). The general reaction catalyzed by GalTs is as follows:

$$\text{UDP-D-galactose} + \text{acceptor} \longrightarrow \text{galactose-acceptor} + \text{UDP}$$

Galactosyltransferases (GalTs) catalyze the transfer of the galactose moiety of activated nucleotide sugars to acceptor substrates (Hennet, 2002; Berger and Rohrer, 2003). These enzymes are involved in a variety of galactosylation reactions of structural and cell wall polysaccharides in all organisms. GalTs are essential enzymes for capsular polysaccharide and lipopolysaccharide O-antigen production in bacteria (Kolkman *et al*., 1997; Guan *et al*., 2001; Watanabe *et al*., 2002; Saksouk *et al*., 2005). In terrestrial plants, they are necessary for plant cell wall biosynthesis for e.g. synthesis of the pectic-galactan cell wall constituents such as homogalacturonan (HGA), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II) (Konishi *et al*., 2004). Similarly, in plants, they are also required in the formation of several other cell wall polysaccharides for e.g. galactomannans and arabinogalactans which lends structural integrity (Edwards *et al*., 1999; Liang *et al*., 2010). In marine red algae, GalTs (α-1,4- and β-1,3-galactosyltransferases) are also required for sulfated galactan (agaran and carrageenan) biosynthesis. These polymers consist of alternating α-1,4-L- and β-1,3-D-galactose(agarans) and α-1,4-D- and β-1,3-D-galactose residues (carrageenans), implying α-

and β-galactosyltransferase involvement (Rees, 1961a, 1961b; Rees, 1969; McCandless and Craigie, 1979; Genicot-Joncour *et al*., 2009). Although GalTs have been isolated and characterized from various bacterial and eukaryotic sources, all attempts have thus far failed to yield GalT homologues from red algae (Lluisma and Ragan, 1997; Weber *et al*., 2004; Collén *et al*., 2006; Lee *et al*., 2007; Pomin and Mourão, 2008; Pomin, 2009b). Heterologous expression systems of GalTs (eukaryotic and bacterial) is well established and multiple platforms have been reported for successful recombinant GalT expression (in *E. coli*, *S. cerevisiae* and insect cell lines) (Nakazawa *et al*., 1993; Hermann *et al*., 1995; Malissard *et al*., 1999; Park *et al*., 2002; Shimma *et al*., 2006; Ko *et al*., 2008; De Pourcq *et al*., 2010). Genetic manipulations for the aforementioned organisms are well established, therefore these organisms are the method of choice for expression of many eukaryotic genes (Romanos, 1992; Baneyx, 1999; Yesilirmak and Sayers, 2009).

In this study, we screened a cDNA library constructed from the agar-producing red algae *Gelidium pristoides* for GalTs involved in sulfated galactan production. We employed an approach where *E. coli* and *S. cerevisiae* mutants deficient in UDP-galactose 4-epimerase (UGE*, EC 5.1.3.2) were transformed with cDNA clones from the library. UGE catalyzes the final step in the Leloir pathway, i.e. the dynamic inter-conversion between UDP-D-galactoseto UDP-D-glucose (Fig.2.1.) (Frey, 1996; Holden *et al*., 2003). Loss-of-function (in UGE mutants) causes galactose toxicity due to accumulating UDP-D-galactose. We exploited this metabolic deficiency created in *E. coli GalE** and *S. cerevisiae* GAL10* mutants to isolate cDNA clones representing putative galactan-associated galactosyltransferase genes. GalT complementation in *GalE* and GAL10 mutants is expected to result in utilization of accumulated UDP-D-galactose and/or GDP-L-galactose. In the event of complementation from galactan synthesizing enzymes, a GDP-mannose 3',5'-epimerase (GME, EC 5.1.3.18) gene which produces GDP-L-galactose, was integrated into the *S. cerevisiae* Gal10 genome. Although this screening methodology was unsuccessful in obtaining a GalT from *G. pristoides,* the study yielded the first UGE enzyme described for red seaweed. This enzyme showed affinity for UDP-D-galactose, similar to other UDP-galactose 4-epimerases.

*Accepted shorthand notation for UDP-galactose 4-epimerase is GALE or UGE for genes derived from bacteria and plant tissues, respectively. The term GAL10 reserved for homologues yeasts and fungi. To avoid confusion the terms GALE and GAL10 will refer to *E. coli* and *S. cerevisiae* mutants only. UGE will refer to plant and red algae UDP-galactose 4-epimerase enzymes. However, the term UDP-galactose 4-epimerase or UGE will also be used when referring to these enzymes collectively.

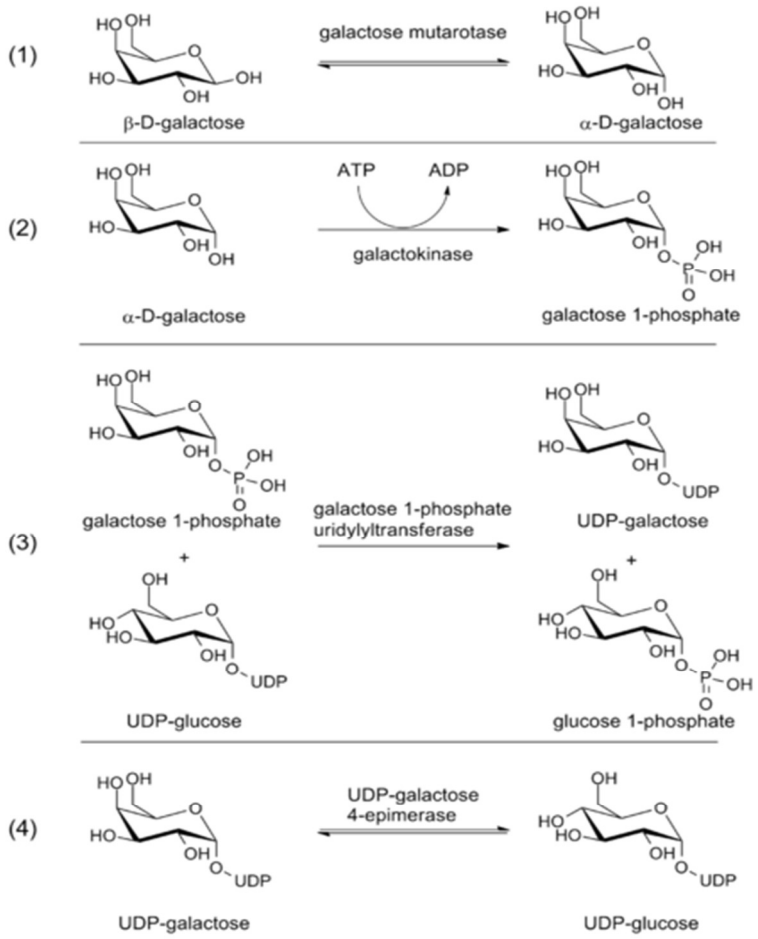

Fig. 2.1. Depiction of the Leloir pathway. The pathway encompasses the sequential enzymatic conversion of UDP-D-glucose to UPD-D-galactose and vice versa (Frey, 1996). The putative Leloir enzymes are as follows: (1) galactosemutarotase (EC 5.1.3.3) (2) galactokinase (EC 2.7.1.6) (3) galactose-1-phosphate uridylyltransferase (EC 2.7.7.12) (4) UDP-galactose 4-epimerase (EC 5.1.3.2).

2.2. MATERIALS AND METHODS

2.2.1. Sulfated polysaccharides extraction. *Gelidium pristoides* (Turner) Kützing (Kalk Bay, Cape Town, South Africa) was identified and confirmed by Prof. Gavin Maneveldt (Department of Biodiversity and Conservation Biology, University of the Western Cape, Cape Town, South Africa). Whole *Gelidium pristoides* plants (Kalk Bay, Cape Town, South Africa) was washed overnight in 100 % acetone and dried at 100 ^{o}C (overnight) before being ground to fine powder. Agar was extracted from 50 g ground tissue with 500 ml dH_2O, pH 6.0 at 37 ^{o}C overnight. The extraction solution was filtered through Miracloth (Merck, Germany) and agar precipitated using 3 volumes absolute ethanol for 1 hour. The precipitate was centrifuged (9000 *g*, 15 min, RT), dried (100 ^{o}C) and ground to fine powder.

2.2.2. Anion exchange separation of extracted sulfated polysaccharides. Two grams of agar powder was dissolved in 50 ml dH_2O pH 8.0 at RT. The residual polysaccharide was removed by centrifugation at 8000 *g* for 5 min. The supernatant was filter sterilized and applied to 5 ml DEAE-Sepharose HiTrap ff column (GE Healthcare, USA) with running buffer [20 mM 1,3-Diaminopropane, pH 8.4] on AKTA prime FPLC system (Amersham Biotech, USA) with a constant flow rate of 5 ml/ min. Charged polysaccharide was eluted over a linear gradient from 0 to 100 % with 100 ml elution buffer [20 mM 1,3-Diaminopropane, pH 8.4; 3 M NaCl]. Individual fractions (2 ml) were collected before and after elution (1 to 50 fractions) and assessed for the presence of sugars using Phenol/ Sulfuric acid assay method with modification (Masuko *et al*., 2005). In a micro titre plate (Greiner Bio-one, Germany) 25 µl sample was added to 25 µl 5 % Phenol (w/v) (final concentration 0.625 %) and 125 µl 37 % Sulphuric acid (H_2SO_4) (v/v) (final concentration 23 %) in 200 µl. The solution was incubated for 5 min and absorbance detected at 492 nm. Separated anionic fractions were subjected to native PAGE electrophoresis on an 8 % polyacrylamide gel (Sigma-Aldrich, Germany) prepared with 0.015 M Sodium tetraborate ($Na_2B_4O_7$), pH 9.1. Electrophoresis was conducted in 0.015 M Sodium tetraborate ($Na_2B_4O_7$), pH 9.1 buffer and visualized by staining with Toludine blue staining solution [0.1 % Toludine blue (w/v); 1 % Glacial acetic acid (v/v)] for 1 hour. Gels were de-stained with 1 % Glacial acetic acid (v/v) until polysaccharides were visible.

2.2.3. RNA extractions from *Gelidium pristoides*. *G. pristoides* plants (Kalk Bay, Cape Town, South Africa) were harvested (10h00 am) and immediately frozen in liquid nitrogen. Plants were ground to fine powder and total RNA extracted according to the method proposed by Valderrama-Cháirez with slight modification (Valderrama-Cháirez *et al*., 2002). Four to five grams ground frozen tissue was added to 10 ml Extraction buffer [150 mM Tris-HCl, pH 7.5; 2 % Sodium Dodecyl Sulfate (SDS) (w/v); 1 % 2-Mercaptoethanol (v/v); 100 mM EDTA] in a 50 ml tube (Corning, USA) and shaken vigorously. To the suspension, 1.1 ml of 5 M Potassium acetate (CH_3COOHK) (final concentration of 0.55 M) and 2.5 ml absolute ethanol (22 % (v/v) final concentration) were added and the mixture was vortexed for 1 min. The suspension was extracted with 15 ml Chloroform: Isoamyl alcohol (24:1) and centrifuged (16000 *g*, 15 min, 4 ^{o}C). The aqueous phase was transferred to a fresh 50 ml tube and extracted with 15 ml Phenol: Chloroform: Isoamyl alcohol (25:24:1). After centrifugation (24000 *g*, 15 min, 4 ^{o}C) the aqueous phase was transferred to new 50 ml tube and extracted with 15 ml Chloroform: Isoamyl alcohol (24:1) followed by vortexing (1 min) and centrifugation (24000 *g*, 15 min, 4 ^{o}C). The aqueous phase was removed and transferred to a fresh 50 ml tube containing ice cold 12 M LiCl (final concentration of 3 M) and RNA precipitated at – 20 ^{o}C overnight. The precipitated nucleic acid was sedimented by centrifugation (11000 *g*, 45 min, 4 ^{o}C) washed with ice cold 70 % ethanol (v/v) and dried by aspiration. Dried RNA was resuspended in 100 µl DEPC-treated H_2O and treated with Deoxyribonuclease I (RNase free) (Frementas, USA) followed by precipitation with 3 volumes absolute ethanol and $^{1}/_{10}$ volume RNase-free 3 M Sodium acetate ($CH_3COOHNa$) overnight. Precipitated nucleic acid was centrifuged (16000 *g*, 45 min, 4 ^{o}C), washed with 70 % ethanol (v/v) and dried. RNA concentration was determined by Nanodrop spectrophotometer. Integrity of RNA was confirmed by Formamide agarose gel electrophoresis (Sambrook *et al*., 1989).

2.2.4. Construction of the *G. pristoides* cDNA library. *G. pristoides* mRNA was separated from total RNA (680 ng/ µl) using a GenElute™ mRNA Miniprep Kit (Sigma-Aldrich, Germany) and precipitated overnight using $^{1}/_{10}$ volume RNase-free 3 M Sodium acetate ($CH_3COOHNa$), $^{1}/_{10}$ volume glycogen (Invitrogen, Germany) and 3 volumes absolute ethanol at – 20 ^{o}C. mRNA was recovered by centrifugation (16000 *g*, 1 hour, 4 ^{o}C), washed with 70 % ethanol (v/v), dried and resuspended 10 µl RNase free dH_2O. mRNA was converted to cDNA as per ZAP-cDNA® synthesis specifications (Stratagene, Germany). Completed double-stranded cDNA was restriction digested with *Eco*RI and *Xho*I (Stratagene, Germany)

and size fractionated using 1 % (w/v) Low Melting Point agarose gel electrophoresis (Invitrogen, Germany). Fractionated cDNA fragments 1.0 kb and larger were directionally cloned into pBluescriptSK vector arms (*Eco*RI/*Xho*I) as per manufacturers instructions (Stratagene, Germany). Approximately 2 µl ligation reaction was packaged using Gigapack Gold® phage packaging reaction (as advised by Stratagene) and resulted in the primary phage library. The primary library obtained a titer of 195 pfu/ µl (20 white to zero blue plaques) in 500 µl i.e. 97500 pfu in total. Approximately 300 µl primary library (58500 pfu) was used for library amplification increasing phage titer to 2.6 X 10^7 pfu/ ml. The amplified library comprised a total volume of 160 ml representing 4.16 X 10^{10} pfu. Phage mass excision was performed to obtain the pBluescript-based cDNA library. Collected transformants and subjected to large scale plasmid isolations using Nucleobond Xtra Maxi Plus columns (Macherey-Nagel, Germany). Plasmid DNA concentration (1800 ng/ µl) was quantified using Nanodrop spectrophotometer. Library plasmid DNA was transformed into *E. coli* DH5α competent cells. Transformed *E. coli* cells were inoculated in 2 ml LB (Luria-Bertani) broth [1 % Tryptone (w/v); 0.5 % Yeast extract (w/v); 0.5 % NaCl (w/v) supplemented with Ampicillin (50 µg/ ml)] and grown overnight at 37 °C. Overnight cultures were subjected to Alkaline lysis plasmid preparations and *Eco*RI/*Xho*I (Frementas, USA) restriction digested for assessment of insert sizes (Sambrook *et al*., 1989). Estimated insert sizes ranged between 500 bp – 4 kb (from 36 surveyed clones).

2.2.5. *Gelidium pristoides* cDNA library screening using an *E. coli* UDP-galactose 4-epimerase (GalE) mutant. The *Gelidium pristoides* cDNA library (2 µg) was transformed into *GalE* mutant *E. coli* PL-2 [(Hfr (PO1), *GalE28*(GalS), *λ*⁻, *e14-*, *relA1*, *spoT1*, *thi-1*)] (*E. coli* Genetic Stock Centre (CGSC), Yale University, New Haven, Connecticut, USA) competent cells by electroporation (C = 25 µF; PC = 200 Ohm; V = 2.5 kV for 0.4 cm cuvettes) using Bio Rad Gene Pulser Xcell electroporation system (Bio Rad Industries, USA). Transformations were selected on M9 minimal media 200 ml 5 X M9 salt solution [33.7 mM $Na_2HPO_4.7H_2O$; 22.0 mM KH_2PO_4; 8.55 mM NaCl; 9.35 mM NH_4Cl; 2 % Bacteriological agar (w/v)] supplemented with 0.5 % galactose (w/v), 1 mM $MgSO_4$, 0.3 mM $CaCl_2$ and 50 µg/ ml Ampicillin. Plates were incubated at 37 °C for 1 – 2 days until colonies developed and transformants screened by colony PCR amplification using vector specific primers, M13F (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and M13R (5'-AGCGGATAACAATTTCACACAGG-3'). PCR conditions were as follows; Initial denaturation: 94 °C for 5 min; Denaturation: 94 °C for 1 min; Annealing temperature: 48 °C for 30 sec; Extension: 72 °C for

2 min and Final extension: 72 °C for 5 min. PCR amplifications were performed with BioTaq Polymerase (Bioline, USA) as per instruction from the manufacturer for 25 cycles. Fragment sizes were assessed by agarose gel electrophoresis (Sambrook *et al*., 1989). Positive colonies were inoculated and grown in 2 ml LB (Luria-Bertani) broth [1 % Tryptone (w/v); 0.5 % Yeast extract (w/v); 0.5 % NaCl (w/v) supplemented with Ampicillin (50 µg/ ml)] overnight at 37 °C for small scale plasmid extraction. Plasmid DNA extracted from overnight cultures was prepared according to alkaline lysis procedure (Sambrook *et al*., 1989). Isolated plasmids were retransformed into *E. coli* PL-2 for confirmation of the conferred phenotype. Confirmed positive clones were sequenced using pBluescript vector specific primers T7 (5'-AATACGA CTCACTATAGG-3') and T3 (5'-AATTAACCCTCACTAAAGGG-3') (Central Analytic Facility, Stellenbosch University). Bioinformatic analysis was performed with DNAMAN software (Lynnon Biosoft, Canada).

2.2.6. Establishment of yeast expression cDNA libraries. cDNA was cloned directly from the pBluescript-based library into yeast expression vectors Yeplac-Kan and pPVD-Kan. Library DNA was partially digested with *Eco*RI/*Xho*I (Fermentas, USA) and cloned into the respective expression vectors. Approximately 20 µg of cDNA library plasmid DNA was digested for 10, 30 and 60 min, respectively. Digestion reactions were purified using Qiagen PCR purification columns (Qiagen, USA) as per manufacturers instructions. Partially digested DNA was cloned into YEpLac112-Kan (*Sal*I/*Eco*RI) and pPVD-Kan (*Eco*RI/*Xho*I). *E. coli* DH5α electro-competent cells prepared according BioRad specifications were transformed with ligation reactions via electroporation (C = 25 µF; PC = 200 Ohm; V = 2.5 kV for 0.4 cm cuvettes) (Bio Rad Gene Pulser system, Bio Rad Industries). Transformants were grown on Luria-Bertani agar plates [1 % Tryptone (w/v); 0.5 % Yeast extract (w/v); 0.5 % NaCl (w/v); 2 % Bacteriological agar (w/v) supplemented with Kanamycin (25 µg/ ml)] for overnight at 37 °C. Libraries size was estimated at 18000 and 12000 clones for the YEpLac- and pPVD-libraries, respectively. Collected transformants were subjected to large scale plasmid isolations using Nucleobond Xtra Maxi Plus according to manufacturers procedure (Macherey-Nagel, Germany). Plasmid DNA was retransformed into *E. coli* DH5α and randomly selected clones subjected to colony PCR amplification for insert size assessment using vector specific primers, M13F (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and M13R (5'-AGCGGAT AACAATTTCACACAGG-3') for YEplac-library clones. PCR was performed as follows; Initial denaturation: 94 °C for 5 min; Denaturation: 94 °C for 1 min; Annealing temperature: 48 °C for 30 sec; Extension: 72 °C for 2 min and Final extension: 72

^{o}C for 5 min. With respect to the pPVD-library clones colony PCR using PGK Fwd (5'-GGCCACTGTGATCTCCAGAGC-3') and Cyc1 Rev (5'-CCGGCCTGTTTACTCACAGG C-3') primers were as follows; Initial denaturation: 94 ^{o}C for 5 min; Denaturation: 94 ^{o}C for 1 min; Annealing temperature: 43 ^{o}C for 30 sec; Extension: 72 ^{o}C for 2 min; Final extension: 72 ^{o}C for 5 min. PCR amplifications were performed with BioTaq Polymerase (Bioline, USA) as per manufacturers instructions for 25 cycles and separated by agarose gel electrophoresis (Sambrook *et al*., 1989). Both libraries contained approximately 80 % inserts ranging from 500 bp to 4 kb and 500 bp to 6 kb, respectively.

2.2.7. Construction of *Saccharomyces cerevisiae* UDP-galactose 4-epimerase (Gal10) knock-out mutant. *S. cerevisiae* Gal10 gene was PCR amplified using Epimerase Fwd (5'-CCGGCACATCTGCGTTTCAGGAACGC-3') and Epimerase Rev (5'-GGATGAGCCTTC GCTCAACAGTGC-3') primers. PCR conditions were as follows; Initial denaturation: 95 ^{o}C for 2 min; Denaturation: 94 ^{o}C for 1 min; Annealing temperature: 55 ^{o}C for 1 min; Extension: at 72 ^{o}C for 2 min; Final extension: 72 ^{o}C for 5 min. PCR reactions were performed for 30 cycles with 2 units of *Pfu* DNA polymerase (Fermentas, USA) per reaction. The 2.5 kb PCR amplified fragment was cloned into pGEM-T Easy (Promega, USA) following manufacturers instructions. Ligation reactions were transformed into *E. coli* DH5α competent cells and selected on Luria agar (Luria-Bertani) [1 % Tryptone (w/v); 0.5 % Yeast extract (w/v); 0.5 % NaCl (w/v); 2 % Bacteriological agar (w/v) supplemented with Ampicillin (50 µg/ ml), 0.1 mM IPTG (Fermentas, USA) and X-Gal (Fermentas, USA)] and grown overnight at 37 ^{o}C. Positive colonies were subjected to small scale plasmid preparations (Sigma-Aldrich, Germany) and sequenced using vector specific M13F and M13R primers (Central Analytic Facility, Stellenbosch University). Gene disruption was achieved by insertion of a geneticin (G418) resistance (Gen^{r}) cassette into a *Stu*I site of the PCR amplified Gal10 gene. Wildtype *S. cerevisiae* YHUM272 [*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG*] was transformed with the linear (100 µg) disrupted Gal10 transformed using "The Best Method" (Gietz and Woods, 2002). Transformants were selected for homologous recombination on SC Dropout media [0.67 % Yeast Nitrogen Base (w/v); 2 mM Leucine; 0.4 mM Tryptophan; 0.3 mM Histidine; 0.2 mM Uracil; 2 % Bacteriological agar (w/v); pH 5.6 supplemented with 50 µg/ ml geneticin (G418)]. Transformed cells were incubated at 30 ^{o}C for 5 – 7 days until colonies developed. Geneticin resistant colonies were replica plated onto fresh SC Drop out/ geneticin media and YP-galactose agar [1 % Yeast extract (w/v); 2 % Peptone (w/v); 2 % galactose (w/v); 2 % Bacteriological agar (w/v); 3 % glycerol (v/v) supplemented with 50 µg/

ml geneticin (G418)]. Colonies were incubated at 30 °C for 5 – 7 days. Cells exhibiting galactose toxicity was selected and screened by PCR using geneticin gene specific primers, Gen Fwd (5'-GATAATGTCGGGCAATCAGG-3') and Gen Rev (5'-AGATCCTGGTATCG GTCTGC-3'), for confirmation of gene insertion. PCR amplifications were performed according to KAPA Taq instructions (KAPA Biosystems, South Africa) and conditions were as follows; Initial denaturation: 94 °C for 2 min; Denaturation: 94 °C for 1 min; Annealing temperature: 55 °C for 1 min; Extension: at 72 °C for 2 min; Final extension: 72 °C for 5 min. Mutant cells were tested for galactose tolerance by minimum inhibitory concentrations (MIC) on YP-galactose agar [1 % Yeast extract (w/v); 2 % Peptone (w/v); 2 % Bacteriological agar (w/v); 3 % glycerol (v/v) supplemented with 50 µg/ ml geneticin (G418)] supplemented with various galactose concentrations ranging from 0.01 % – 0.5 % (w/v). 0 % galactose used as control.

2.2.8. Cloning of *Arabidopsis thaliana* GDP-mannose 3', 5'-epimerase (GME) and integration into the *Saccharomyces cerevisiae* Gal10 mutant. RNA extracted from *A. thaliana* was converted to single-strand cDNA using the Superscript® First Strand Synthesis System (Invitrogen, Germany) according to the manufacturers instructions. Single stranded cDNA was used as template for PCR amplification of the *A. thaliana* GDP-mannose 3', 5'-epimerase (*At*GME) gene. PCR amplification was performed using primers GDP-Epi Fwd (5'-GGCCGAATTCATGGGAACTACCAATGAACA-3') and GDP-Epi Rev2 (5'-GGCCA GATCTTCACTCTTTTCCATCAGC CGC-3') containing restriction sites *Eco*RI and *Bgl*II, respectively. PCR conditions were as follows: Initial denaturation: 95 °C for 2 min; Denaturation: 95 °C for 1 min; Annealing: 55 °C for 30 sec; Extension: 72 °C for 2 min; Final extension: 72 °C for 5 min. Amplification reactions were performed for 30 cycles with 2 units of *Pfu* DNA polymerase (Fermentas, USA). The amplified 1.134 kb *At*GME fragment was subsequently cloned into the pGEM-T Easy vector (Promega, USA). Ligation reactions were transformed into *E. coli* DH5α competent cells and selected on Luria agar (Luria-Bertani) [1 % Tryptone (w/v); 0.5 % Yeast extract (w/v); 0.5 % NaCl (w/v); 2 % Bacteriological agar (w/v) supplemented with Ampicillin (50 µg/ ml), 0.1 mM IPTG (Fermentas, USA) and X-Gal (Fermentas, USA)] and grown overnight at 37 °C. Positive colonies were subjected to small scale plasmid preparations (Sigma-Aldrich, Germany) and sequenced using vector specific M13F and M13R primers (Central Analytic Facility, Stellenbosch University). The *At*GME gene was excised using *Eco*RI/*Bgl*II restriction digestion (Fermentas, USA) and cloned into yeast integration vector pCEL13 digested with *Bam*HI and *Eco*RI (Fermentas, USA). To

create *S. cerevisiae* Gal10/GME cell lines *S. cerevisiae* Gal10 was transformed with linearized pCEL13/*At*GME (*Eco*RI). *S. cerevisiae* Gal10 competent cells were transformed according to the "The Best method" (Gietz and Woods, 2002). Transformants were selected on SC Dropout media [0.67 % Yeast Nitrogen Base (w/v); 2 mM Leucine; 0.3 mM Histidine; 0.4 mM Tryptophan; 2 % Bacteriological agar (w/v); pH 5.6 without Uracil supplemented with 50 µg/ ml geneticin (G418)]. Transformations were incubated for 2 – 5 days to allow for colony development. Transformants were selected for Uracil prototrophy and positive colonies screened for successful integration by diagnostic PCR using GDP-Epi Fwd and GDP-Epi Rev2 primers. PCR conditions were as follows: Initial denaturation: 94 ^{o}C for 2 min; Denaturation: 94 ^{o}C for 1 min; Annealing: 55 ^{o}C for 30 sec; Extension: 72 ^{o}C for 2 min; Final extension: 72 ^{o}C for 5 min. PCR amplification was performed for 30 cycles with 2 units of KAPA Taq DNA polymerase (KAPA Biosystems, South Africa).

2.2.9. Screening of the *Gelidium pristoides* yeast expression cDNA library using the *S. cerevisiae* Gal10/GME cells. *S. cerevisiae* Gal10/GME cells were transformed with yeast expression cDNA libraries by electroporation (C = 25 µF; PC = 200 Ohm; V = 2.5 kV for 0.4 cm cuvettes) (Bio Rad Gene Pulser Xcell system, Bio Rad Industries). Competent cells were prepared as described in Current Protocols (Current Protocols in Molecular Biology, Unit 13.7). Yeast cells transformed with YEpLac112-library were screened on SC Dropout media [0.67 % Yeast Nitrogen Base (w/v); 0.02 % galactose (w/v); 3 % glycerol (v/v); 2 mM Leucine; 0.3 mM Histidine; 2 % Bacteriological agar (w/v); pH 5.6 supplemented with 50 µg/ ml geneticin (G418)] and [0.67 % Yeast Nitrogen Base (w/v); 0.02 % galactose (w/v); 3 % glycerol (v/v); 2 mM Leucine; 0.4 mM Tryptophan; 2 % Bacteriological agar (w/v); pH 5.6 supplemented with 50 µg/ ml geneticin (G418)] was used for pPVD-library transformant selection. Transformations were incubated at 30 ^{o}C for 5 – 7 days to allow for colony development.

2.2.10. Complementation studies of *S. cerevisiae* Gal10/GME and *E. coli* PL-2 mutants. The *A. thaliana* UGE1 gene was PCR amplified from cDNA using *At*UGE Fwd (5'-CCGGCTGCAGATGGGTTCTTCTGTGG-3') and *At*UGE Rev (5'-CCGGGAATTCTCAA AGCTTATTCTGGTAACC-3') and cloned into pJET1.2 cloning vector following manufacturers instructions (Fermentas, USA). PCR was performed as follows; Initial denaturation: 95 ^{o}C for 2 min; Denaturation: 98 ^{o}C for 20 sec; Annealing temperature: 55 ^{o}C for 20 sec; Extension: 72 ^{o}C for 30 sec; Final extension: 72 ^{o}C for 5 min. PCR reactions were

performed using KAPA HiFi polymerase (KAPA Biosystems, South Africa) in accordance with the manufacturers. Ligations were transformed into *E. coli* DH5α competent cells and selected on Luria agar (Luria-Bertani) [1 % Tryptone (w/v); 0.5 % Yeast extract (w/v); 0.5 % NaCl (w/v); 2 % Bacteriological agar (w/v) supplemented with Ampicillin (50 µg/ ml)] overnight at 37 ^{o}C. Positive colonies were subjected to small scale plasmid isolations (GeneJET^{TM} Plasmid Miniprep kit, Fermentas, USA) and sequenced using pJET1.2F (5'-CGACTCACTATAGGGAGAGCGGC-3') and pJET1.2R (5'-AAGAACATCGATTTTCCA TGGCAG-3') vector specific primers (Central Analytic Facility, Stellenbosch University). The *A. thaliana* UGE1 PCR fragment was excised from pJET-*At*UGE and simultaneously ligated into pYES2.0 (*Xho*I/*Xba*I) (Invitrogen, Germany) and pRSET-C (*Pst*I/*Eco*RI) (Invitrogen, Germany), respectively (Sambrook *et al*., 1989). Ligations reactions were transformed into *E. coli* DH5α competent cells and transformants selected on Luria agar (Luria-Bertani) [1 % Tryptone (w/v); 0.5 % Yeast extract (w/v); 0.5 % NaCl (w/v); 2 % Bacteriological agar (w/v) supplemented with Ampicillin (50 µg/ ml)] and grown overnight at 37 ^{o}C. Positive colonies were inoculated into Luria broth (Luria-Bertani) [1 % Tryptone (w/v); 0.5 % Yeast extract (w/v); 0.5 % NaCl (w/v); 2 % Bacteriological agar (w/v) supplemented with Ampicillin (50 µg/ ml)] and grown overnight at 37 ^{o}C. Overnight cultures were subjected to small scale plasmid preparations (GeneJET^{TM} Plasmid Miniprep kit, Fermentas, USA) and confirmed by *Xho*I/*Xba*I (pYES-*At*UGE) and pRSET-*At*UGE clones (*Pst*I/*Eco*RI) restriction digestion (Sambrook *et al*., 1989). The constructs were subsequently individually transformed into *S. cerevisiae* Gal10/GME cells and *E. coli* PL-2, respectively. pYES-*At*UGE was transformed into *S. cerevisiae* Gal10/GME cells by electroporation (C = 25 µF; PC = 200 Ohm; V = 2.5 kV for 0.4 cm cuvettes) using Bio Rad Gene Pulser Xcell system (Bio Rad Industries, USA) and selected on SC Drop out media [0.67 % Yeast Nitrogen Base (w/v); 2 mM Leucine; 0.4 mM Tryptophan; 0.3 mM Histidine; 3 % glycerol (v/v); 2 % Bacteriological agar (w/v), pH 5.6 supplemented with 50 µg/ ml geneticin (G418)]. Transformed *S. cerevisiae* Gal10/GME cells were incubated at 30 ^{o}C for 2 – 5 days to allow for colony development. Similarly, pRSET-*At*UGE was introduced into *E. coli* PL-2 by electroporation (C = 25 µF; PC = 200 Ohm; V = 1.8 kV for 0.1 cm cuvettes) and selected on M9 minimal media 200 ml 5 X M9 salt solution [33.7 mM $Na_2HPO_4.7H_2O$; 22.0 mM KH_2PO_4; 8.55 mM NaCl; 9.35 mM NH_4Cl; 2 % Bacteriological agar (w/v)] supplemented with 0.5 % galactose (w/v), 1 mM $MgSO_4$, 0.3 mM $CaCl_2$ and 50 µg/ ml Ampicillin. Transformations were incubated at 37 ^{o}C for 1 – 2 days until colonies developed.

2.2.11. Plasmid preparations from *Saccharomyces cerevisiae.* Positive transformants were inoculated in 5 ml selection media [0.67 % Yeast Nitrogen Base (w/v); 0.02 % galactose (w/v); 2 mM Leucine; 0.4 mM Tryptophan; 0.3 mM Histidine or 0.2 mM Uracil; pH 5.6 supplemented with 50 µg/ ml geneticin (G418)]. Cultures were grown at 30 ^{o}C for 1 – 2 days and sedimented by centrifugation (6000 *g*, 5 min, 4 ^{o}C). Cells were resuspended in (100 µl) 67 mM KH_2PO_4, pH 7.5 buffer and 10 µl (25 µg/ µl) Lyticase (Sigma-Aldrich, Germany) and incubated at 37 ^{o}C for 1 hour. Cells were lysed with 200 µl Lysis solution [1 % SDS (v/v); 200 mM NaOH] was added and mixed by gentle inversion. Cell debris was precipitated with 350 µl Neutralization solution [3 M Potassium acetate (CH_3COOK); 11.5 % Glacial acetic acid (v/v)]. Precipitate was sedimented by centrifugation (16000 *g*, 5 min, 4 ^{o}C). Supernatant was removed and plasmid DNA precipitated with $^{1}/_{10}$ volume 3 M Sodium acetate ($CH_3COOHNa$) and 3 volumes ice cold absolute ethanol (30 min at – 20 ^{o}C). Precipitated DNA was recovered by centrifugation (16000 *g*, 5 min, 4 ^{o}C) and washed with 70 % ethanol (v/v), dried and resuspended in 1 X T. E buffer [10 mM Tris-HCl; 1 mM EDTA; pH 8.0]. Plasmids were transformed into *E. coli* DH5α competent cells to facilitate bulk plasmid extractions.

2.2.12. Genomic DNA isolation from *Saccharomyces cerevisiae*. *S. cerevisiae* genomic was prepared according to Harju and co-workers with slight modification (Harju *et al*., 2004). Selected colonies were inoculated into selection media [0.67 % Yeast Nitrogen Base (w/v); 2 mM Leucine; 0.3 mM Histidine; 0.4 mM Tryptophan; 2 % Bacteriological agar (w/v); pH 5.6 without Uracil supplemented with 50 µg/ ml geneticin (G418)] and grown for 20 - 24 hrs at 30 °C. Cultures were harvested by centrifugation (13000 g, 5 min, RT). Cells were resuspended in 200 µl of Harju- buffer [10 mM Tris-HCl, pH 8.0; 100 mM NaCl; 1 mM EDTA; 2 % (v/v); Triton X-100 (v/v); 1 % SDS (v/v)] and transferred to 1.5 ml eppendorff tubes. Tubes were immersed in liquid N2 for 2 minutes and the frozen cell suspensions were placed at 95 °C for 1 min. The last two steps were repeated twice after which 200 µl Chloroform was added. The extraction was vortexed and centrifuged (13000 g, 5 min, RT). The upper aqueous phase was transferred to fresh eppendorf tubes each containing 400 µl ice-cold absolute ethanol and mixed by gentle inversion. DNA was precipitated at -20 °C and harvested by centrifugation (10000 g, 10 min, 4 °C). DNA pellets was washed with 70 % ethanol (v/v), air dried and dissolved in 50 µl T.E. buffer [10 mM Tris-HCl, pH 8.0; 1 mM EDTA]

2.2.13. Genomic DNA preparation and Southern hybridization for determination of *Gelidium pristoides* UDP-galactose 4-epimerase (*Gp*UGE) copy number. Genomic DNA extractions were performed using sample preparations after RNA was recovered. Genomic DNA was precipitated with addition of 3 volumes of absolute ethanol at – 20 ^{o}C for 1 hour. Coiled genomic DNA was spooled off with a sterile glass pipette and washed with 70 % ethanol (v/v), dried and resuspended in 1 X T. E. buffer [10 mM Tris-HCl; 1 mM EDTA, pH 8.0]. Genomic DNA concentration determination was performed using a Nano Drop spectrophotometer. *G. pristoides* genomic DNA (10 µg) was digested with *Bam*HI/*Hind*III (Fermentas, USA) overnight and DNA fragments separated by agarose gel electrophoresis (Sambrook *et al*., 1989). After separation the DNA was depurinated with 0.125 M HCl for 20 min and denatured with 0.4 M NaOH. Denatured nucleic acid was alkaline transferred [0.4 M NaOH] overnight to positively charged nylon (Roche Diagnostics, South Africa) by downward capillary method. Pre-hybridization (Roche Diagnostics, South Africa) of nylon membranes were performed for 30 min at 40 ^{o}C. *Gp*UGE DNA probe (prepared according to Roche instructions) was denatured for 5 min and snap-cooled on an ice-ethanol bath. Nylon membranes were incubated overnight with DNA probe/pre-hybridization solution (Roche Diagnostics, South Africa) at 40 ^{o}C. Membranes were washed with 2 X SSC (2 X 15 min) at room temperature followed by 0.1 X SSC stringency washes (2 X 15 min) at 60 ^{o}C. Detection of DNA fragments were executed as per instructions from Roche (Roche Diagnostics, South Africa).

2.2.14. Protein preparation from *E. coli* PL-2 overexpressing *Gelidium pristoides* UDP-galactose 4-epimerase (Clone 17). Overnight *E. coli* PL-2 cultures harbouring library Clone 17 plasmid DNA was grown in 5 ml LB (Luria-Bertani) broth [1 % Tryptone (w/v); 0.5 % Yeast extract (w/v); 0.5 % NaCl (w/v) supplemented with Ampicillin (50 µg/ ml)] and used to inoculate 100 ml of fresh SOB broth [2 % Tryptone (w/v); 0.5 % Yeast extract (w/v), 8.5 mM NaCl; 2.5 mM KCl; 10 mM $MgSO_4$; 10 mM $MgCl_2$; pH 7.0] to OD_{600} = 0.1. *E. coli* cultures were grown for an additional 4 hours until the early-exponential phase (OD_{600} = 0.4 – 0.5) was reached and expression induced with 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) (Fermentas, USA). Cells were grown for 6 hours and harvested by centrifugation (5000 *g*, 5 min, 4 ^{o}C). Crude protein was extracted from *E. coli* PL-2 (Clone 17) cultures by resuspending cells in 1 ml protein extraction buffer [50 mM Potassium

phosphate buffer, pH 7.5; 2 mM DTT; 0.1 % Triton X-100 (v/v); $^1/_2$ X Protease inhibitor cocktail tablet (Sigma-Aldrich, Germany)]. Cells were lysed by three freeze-thaw cycles followed by three 10 sec sonication cycles with intermittent cooling on ice. Cell debris was removed by centrifugation (16000 *g*, 15 min, 4 ^{o}C) and supernatant transferred to a fresh 1.5 ml eppendorf tube.

2.2.15. Protein determination and UDP-galactose 4-epimerase activity assays. Protein concentration was determined by method of Bradford using 10 µg/ ul BSA (Bovine Serum Albumin) as a standard (Bradford *et al*, 1976). Proteins were diluted to 10 µg/ µl in buffer [20 mM Tris-HCl, pH 8.5; 0.25 mM NAD^+] prior to activity assays. UDP-galactose 4-epimerase converts UDP-D-galactose to UDP-D-glucose. Activity was measured using UDP-D-glucose dehydrogenase (UGD) as coupling enzyme which converts UDP-D-glucose to UDP-glucuronic acid with subsequent liberation of NADH (Wilson and Hogness, 1964). Epimerase reactions were initiated with addition of 100 µg (10 µl) of total protein to 190 µl standard reaction buffer [20 mM Tris-HCl, pH 8.5; 0.25 mM NAD^+; 2 mM UDP-D-galactose; 0.0125 units UDP-D-glucose dehydrogenase (Sigma-Aldrich)]. Standard epimerase assays were performed at RT (25 ^{o}C) over a linear period of 15 min and net production of NADH continuously measured by following changes in A_{340}. Stoichiometrically, 2 mol NADH is formed upon oxidation of 1 mol UDP-D-glucose.

2.3. RESULTS

2.3.1. *Gelidium pristoides* produces sulfated polysaccharides

Prior to library construction *G. pristoides* was scrutinized for the presence of sulfated polysaccharides. Polymers were extracted and separated into two fractions by anion-exchange chromatography: (i) neutral agarose and (ii) sulfated agar (Figure 2.3.1.A.). The presence of sugars was detected with Phenol/ Sulphuric acid assays and the presence of charged polysaccharide was confirmed by toluidine blue staining after separation by SDS-PAGE (Figure 2.3.1.B.).

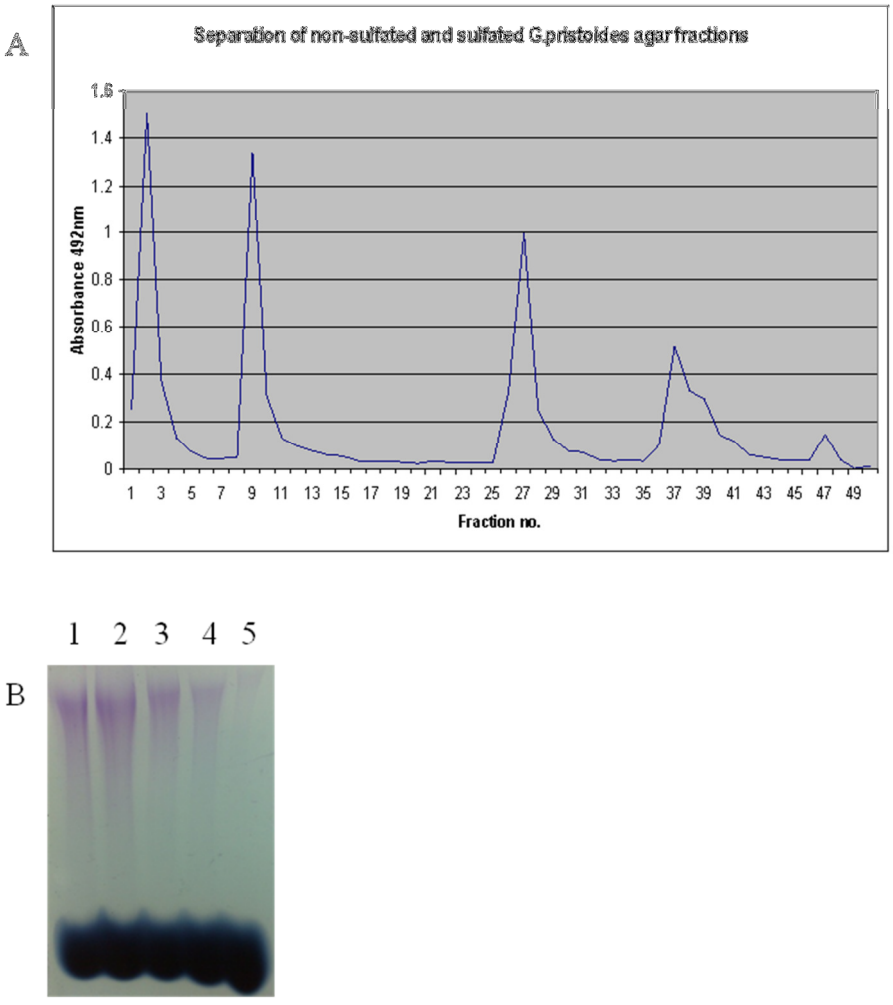

Figure 2.3.1. Separation of *Gelidium pristoides* sulfated polysaccharides into uncharged and charged fractions. Polysaccharide extracts were subjected to anion-exchange chromatography on a DEAE-Sepharose column. (A) Separation was achieved into non-ionic fractions (1-12) and anionic fractions (25-43) as confirmed by the Phenol/ Sulphuric acid assay. Anionic fractions were concentrated into 5 pools to facilitate electrophoresis. Fractions 25, 26, 27 and 28 formed Pool 1. Pool 2 contained fractions 29, 30, 31 and 32. Pool 3 consisted of fractions 33, 34, 35 and 36. Pool 4 (37, 38, 39 and 40), while fractions 41, 4 and 43 formed Pool 5. (B) PAGE gel electrophoresis of pooled anionic fractions (25-43) as visualized by 0.1 % (w/v) toludine blue staining (Lane 1-5).

2.3.2. Construction of *S. cerevisiae* UDP-galactose 4-epimerase (Gal10) mutant.

A PCR amplified *S. cerevisiae* GAL10 gene was disrupted by the insertion of a geneticin resistance cassette (Figure 2.3.2.A.). The interrupted Gal10 gene fragment was integrated into the *S. cerevisiae* chromosome through homologous recombination. Positive transformants

were selected for their inability to grow on galactose containing media, while displaying geneticin resistance. A minimum inhibitory concentration of 0.02 % galactose (w/v) was determined with plate assays. From a total of 180 geneticin resistant colonies, only one exhibited toxicity to galactose. PCR amplification affirmed the presence of the integrated resistance gene (Figure 2.3.2.B.).

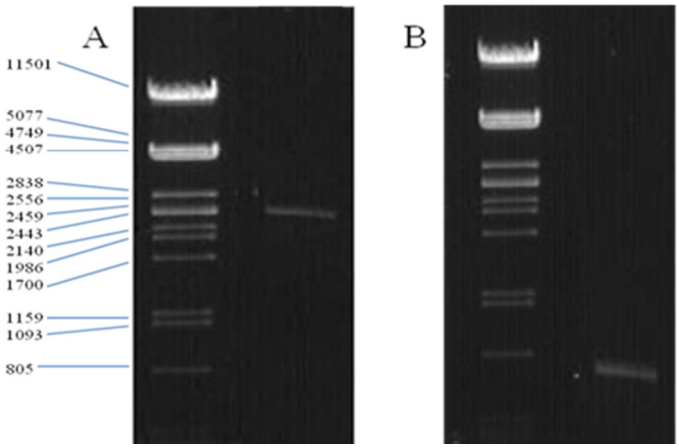

Figure 2.3.2. PCR amplification of *Saccharomyces cerevisiae* Gal10 gene and insertion into wildtype *S. cerevisiae* genome. (A) *S. cerevisiae* Gal10 gene PCR amplified from genomic DNA (approx. 2.5 kb). The Gal10 gene was disrupted by insertion of geneticin resistance cassette and inserted into wildtype *S. cerevisiae* YHUM272, yielding the GAL10 knock-out mutant. (B) PCR confirmation of insertion of geneticin resistance gene into the genome of *S. cerevisiae*.

S. cerevisiae does not naturally produce GDP-L-galactose. To confer the trait of GDP-L-galactose biosynthesis from GDP-D-mannose, a GDP-mannose 3,5-epimerase (GME) gene was introduced into the *S. cerevisiae* Gal10 mutant (Figure 2.3.3.). A GME gene from wildtype *A. thaliana* (NM_122767) was PCR amplified integrated into the *S. cerevisiae* Gal10 genome by plasmid-mediated homologous recombination. *S. cerevisiae* Gal10 (Uracil deficient) was transformed with chromosome integration construct pCEL13-*At*GME resulting in several positive transformants. Several colonies were PCR screened for the presence of the integrated gene and positive transformants selected for library screening.

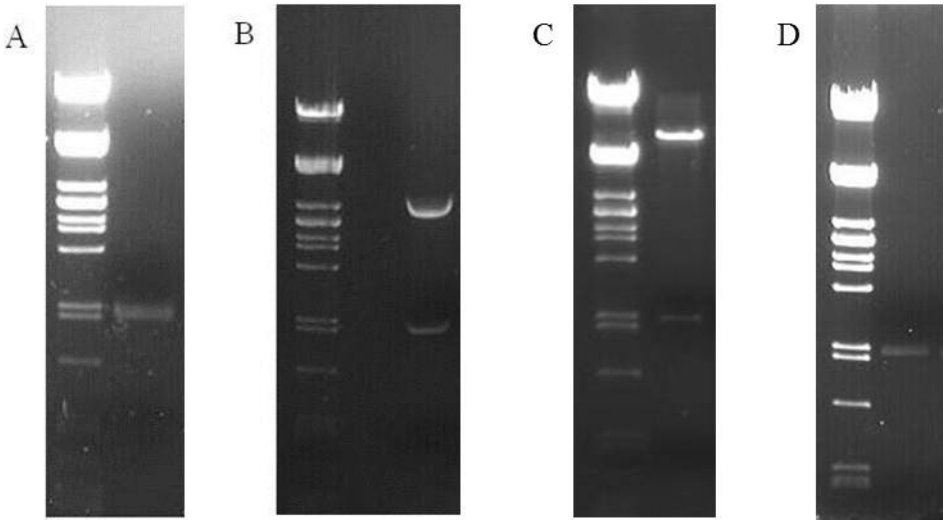

Figure 2.3.3. PCR amplification and cloning of *Arabidopsis thaliana* GDP-mannose 3,5-epimerase (*At*GME). (A) PCR of *At*GME from 3 individual preparations of *A. thaliana* leaf RNA showing amplification of an approximate 1.1 kb fragment (B) *Eco*RI restriction digestion of pGEM-*At*GME clones showing excision of the cloned gene. Subsequent DNA sequencing and analysis confirmed *A. thaliana* GME gene identity (C) Diagnostic restriction digestion (*Bgl*II/*Eco*RI) of pCEL13-*At*GME constructs for *S. cerevisiae* chromosome integration (D) PCR amplification shows the presence of *At*GME gene in *S. cerevisiae* Gal10.

2.3.3. *Gelidium pristoides* cDNA library screening

Mutant complementation as a strategy to screen for galactose metabolizing genes was verified in UGE knock-out mutants, *E. coli* GalE (PL-2) and *S. cerevisiae* Gal10/GME, respectively. An UDP-galactose 4-epimerase 1 (*At*UGE) gene was amplified from *A. thaliana* and expressed in these mutants (Figure 2.3.4.). Recombinant cells were indeed able to recover from galactose toxicity (Figure 2.3.5.A and Figure 2.3.5.B). In order to facilitate screening for galactan biosynthesis genes from *G. pristoides*, three cDNA libraries were constructed. To enable screening in *E. coli* PL-2, cDNA was directionally cloned into pBluescriptSK. Subsequently, cDNAs from the pBluescript primary library was directionally cloned into Yeplac112 and pPVD1 yeast expression vectors. Libraries were transformed into the respective *E. coli* and *S. cerevisiae* UGE knock-out mutants and screened for complementation in the presence of galactose.

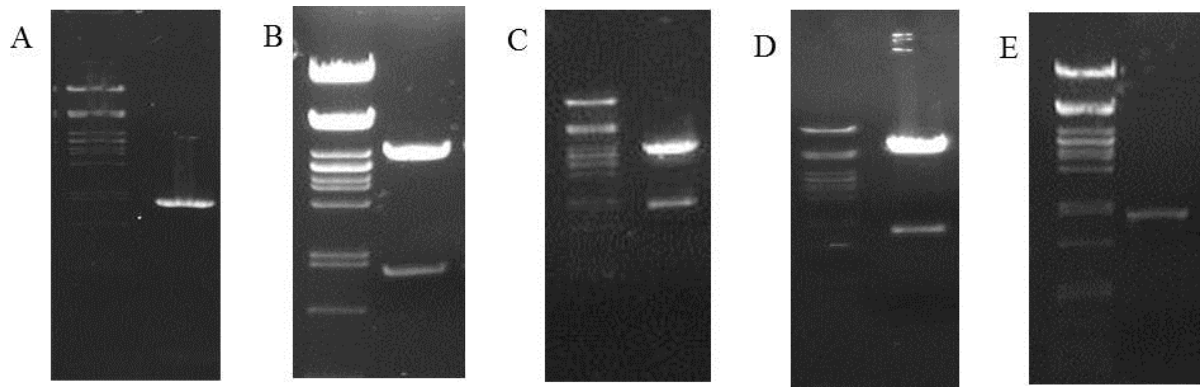

Figure. 2.3.4. Construction of *A. thaliana* UGE vectors for complementation of *S. cerevisiae* Gal10 knock-out mutants. (A) PCR amplification of the *At*UGE gene (B) *Pst*I/*Eco*RI of pJET-*At*UGE (C) Restriction digestion (*Pst*I/*Eco*RI) of completed pRSET-*At*UGE construct (D) Restriction digestion (*Xho*I/*Xba*I) prior to transformation into the *S. cerevisiae* Gal10/GME (E) Diagnostic PCR amplification of a positive pYES-*At*UGE clone.

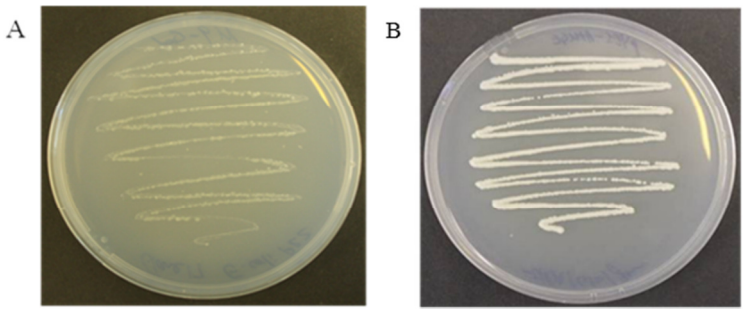

Figure 2.3.5. Complementation studies conducted in *E. coli* and *S. cerevisiae* UDP-galactose 4-epimerase knock-out mutants. Complementation experiments show *Arabidopsis thaliana* UGE (*At*UGE) was able to rescue the galactose toxicity phenotype exhibited by mutant cells. (A) *E. coli* PL-2 cells containing *At*UGE (pRSET-*At*UGE) selected on M9 minimal-galactose media and (B) *At*UGE (pYES-*At*UGE) expressing *S. cerevisiae* Gal10/GME selected on SC Dropout-galactose media

2.3.4. *Gelidium pristoides* UDP-galactose 4-epimerase is a novel discovery

A total of 48 clones were selected from library *E. coli* and *S. cerevisiae* clones with the ability to grow on galactose. Library inserts were 100 % identical on the nucleotide sequence level. BLASTX analysis of coding regions revealed no homology to known GalTs. However, a 65 % similarity to UDP-galactose 4-epimerase from *Phaeodactylum tricornutum* UGE (XP_002185705.1) was evident on the amino acid sequence level. A single plasmid clone (Clone 17) suspected to contain the entire UGE ORF was selected for further study. DNA

sequence analysis showed fragment insert length to be 1444 nucleotides (See Appendix II for nucleotide sequence) including a poly(T)-tail. NCBI Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) indicated the *G. pristoides* UGE open reading frame comprised 1035 nucleotides (345 aa) from start (ATG) to (TAA) stop. Analysis of the coding sequence revealed a (TAG) stop located 174 bp in the 5' untranslated region (UTR) upstream of the (ATG) start codon. Amino acid sequence alignments to *E. coli* (BAA35421), *S. cerevisiae* (EDN64634), *H. sapiens* (NP_001121093) and *A. thaliana* (AAC33955) UGE enzymes indicated *G. pristoides* UGE had an overall homology of 57 % to these known epimerases. Amino acid alignments indicated conserved regions corresponding to GXXGXXG-NAD^+ binding motifs as well as Ser and Tyr-X-X-X-Lys amino acid residues as described for NAD(P) Rossmann-fold epimerase/dehydratases (Figure 2.3.6.) (Thoden *et al*., 2000). UGEs have been cloned from various origins (bacteria, humans and terrestrial plants), but none have been cloned from red algae prior to our study.

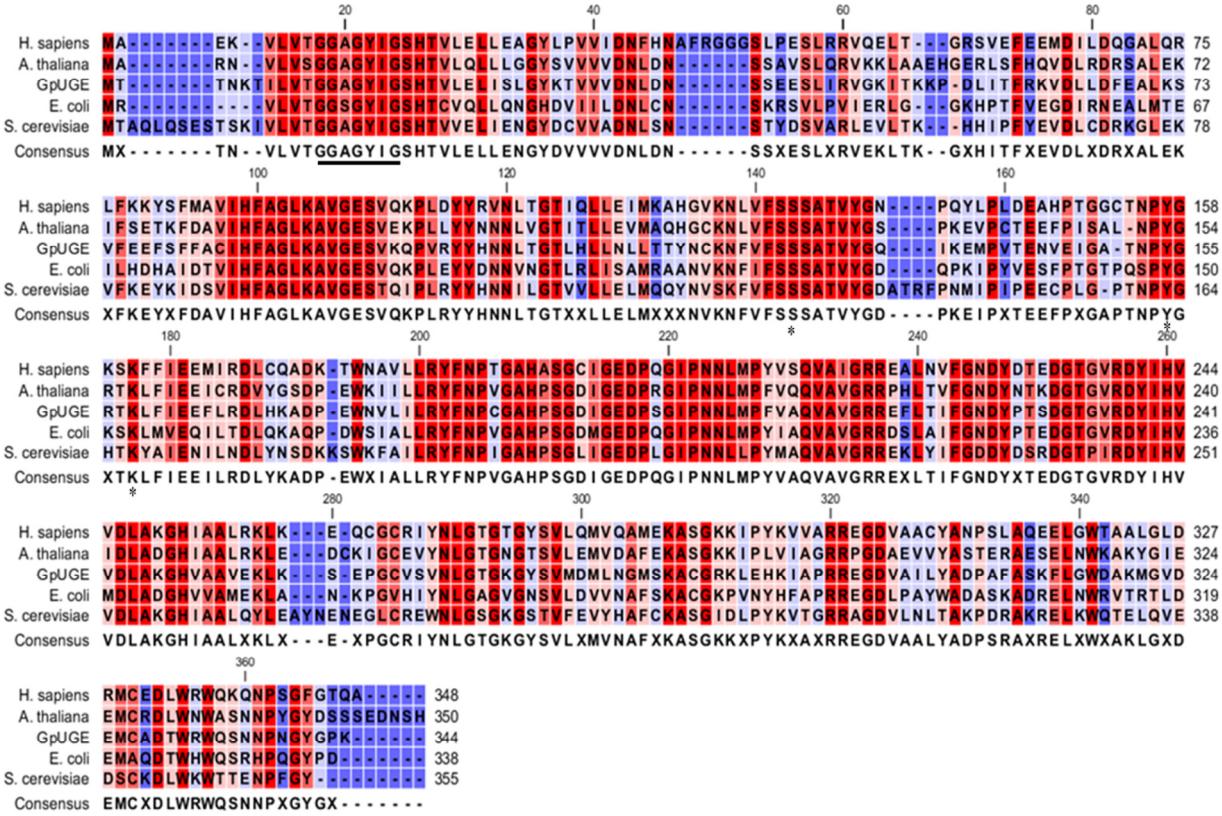

Figure 2.3.6. Amino acid sequence alignment indicating conservancy to known UGE genes. *Gelidium pristoides* UGE compared known sequences from *E. coli* (BAA35421), *S. cerevisiae* (EDN64634), *H. sapiens* (NP_001121093) and *A. thaliana* (AAC33955). Underlined residues highlight conserved GXXGXXG-NAD^+ binding motifs as well as Ser and Tyr-X-X-X-Lys amino acid residues. Multiple alignments (CLC Genomics Workbench, CLC Bio, Denmark) indicate conserved amino acid residues in red. Residues in blue highlight designate non-homologous regions.

2.3.5. Genomic assessment of UDP-galactose 4-epimerase copy number in *Gelidium pristoides*

Southern hybridization analysis detected the presence of two possible UGE gene copies in the *G. pristoides* genome (Figure 2.3.7.). Furthermore, Southern analysis confirmed that the gene originated from *G. pristoides*.

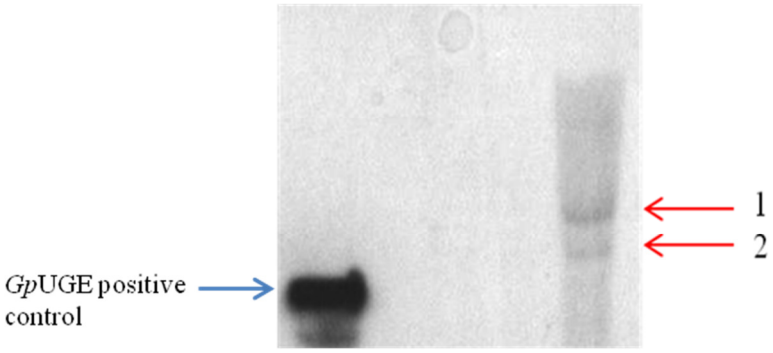

Figure 2.3.7. Southern hybridization detection of two UGE gene copies in the *Gelidium pristoides* genome. *G. pristoides* genomic DNA (10 µg) digested with *Bam*HI/*Hind*III was alkaline transferred (with 0.4 M NaOH) to a positively charged nylon membrane and hybridized overnight (40 °C) with a homologous *Gelidium pristoides* UGE probe. Two distinct signals were detected possibly indicating the presence of two UGE copies in the *G. pristoides* genome, although only one isoform was isolated during library screening.

2.3.6. *G. pristoides* UGE is a functional enzyme

The cloned *Gp*UGE was assessed for activity with UDP-D-glucose dehydrogenase (UGD) coupled-assays. The enzyme showed marked ability to convert UDP-D-galactose to UDP-D-glucose compared to the control (*E. coli* PL-2 crude protein extract) (Figure 2.3.8.). No kinetic lag was observed upon initiation of activity as described for *Kluyveromyces fragilis* UGE (Nayar *et al*., 2004). Denatured *E. coli* PL-2 crude extracts were unable to convert UDP-D-galactose in comparison to intact enzyme used as control, demonstrating epimerase activity (Figure 2.3.9.).

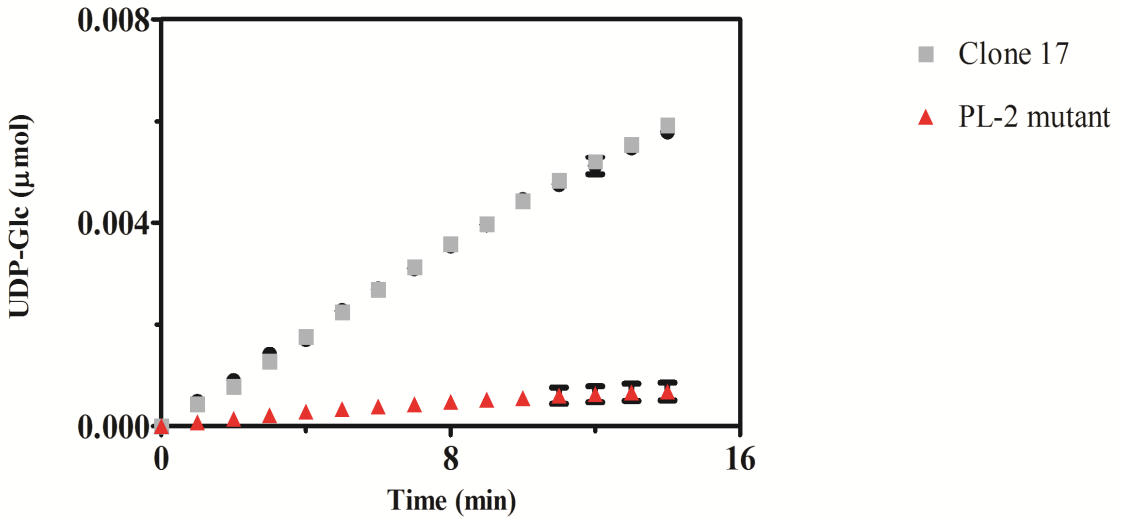

Figure 2.3.8. Preliminary assessment of *Gelidium pristoides* UDP-galactose 4-epimerase activity. Cellular extracts from library isolate (Clone 17) and control cells harbouring empty vector (pBluescriptSK) were assessed for epimerase activity in the presence of 2 mM UDP-D-galactose. The ability of *Gelidium pristoides* UGE to epimerase UDP-D-galactose to UDP-D-glucose was assessed with coupling enzyme UDP-D-glucose dehydrogenase under standard reaction conditions. The UDP-D-glucose dehydrogenase catalyzed reaction results in production of 2 mol NADH (detected at 340 nm) for every 1 mol UDP-D-glucose consumed. UDP-glucuronic acid is produced as a by-product. An increase in NADH production is observed as an indication of UGE activity. *G. pristoides* UGE (Clone 17) in the presence of 2 mM UDP-D-galactose exhibited a linear increase in activity over time (15 min) versus insignificant change in absorbance exhibited in *E. coli* PL-2 control. Represented data are means ± S.E.M. of 6 replicates.

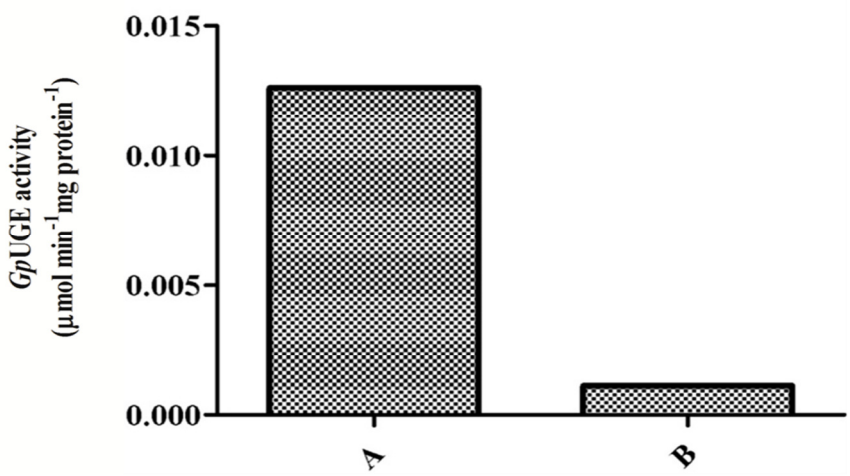

Figure 2.3.9. *Gelidium pristoides* UDP-galactose4-epimerase (UGE) activity. (A) Non-denatured *Gelidium pristoides* UGE (B) boiled *Gelidium pristoides* UGE *E. coli* crude extract. Denatured enzyme was unable to convert UDP-D-galactose to UDP-D-glucose, in contrast to non-denatured *Gelidium pristoides* UGE crude extract. Epimerase reactions were initiated with (100 µg total protein) and were performed under standard conditions for Assay II.

2.4. DISCUSSION

Gelidium pristoides, an agar-producing red algae, was identified in Kalk Bay (Cape Town, South Africa). Expression cDNA libraries constructed from *Gelidium pristoides* mRNA were screened for galactan (agar) biosynthesis-associated genes through functional complementation of *E. coli* and *S. cerevisiae* UDP-galactose 4-epimerase (UGE) mutants. These mutants are unable to metabolize UDP-D-galactose as accumulation of this nucleotide sugar causes galactose toxicity. In eukaryotic and prokaryotic UGE knock-out mutants, severe cell wall defects have been documented due to the substrate being unavailable for galactosylation (Dörmann and Benning, 1998; Seifert *et al*., 2002; Mozzi *et al*., 2003; Seifert 2004; Barber *et al*., 2005; El-Ganiny *et al*., 2010). The complementation strategy was based on the rationale that genes potentially involved in galactan biosynthesis, such as GalTs or UGEs might utilize accumulating UDP-D-galactose for galactosylation, thereby relieving UGE mutants deprived of cellular phosphate through release of UDP. This screening system however did not yield any GalT cDNAs in the heterologous hosts (an approximate 240000 *S. cerevisiae* and *E. coli* colonies were cumulatively screened yielded. A possible explanation is that galactosyltransferase activity (galactosylation) in *S. cerevisiae* requires both co-expression of UDP-galactose 4-epimerase and UDP-D-galactose transporter activity to be functional (Wildt and Gerngross, 2005). In *E. coli*, membrane preparations of a UGE deficient strain expressing *Klebsiella pneumonia* galactosyltransferase (WbbO) has been shown to transfer ^{14}C-galactose from UDP-[^{14}C]-galactose to undecaprenyl pyrophosphoryl-GlcNAc (Guan *et al*., 2001). Although these authors have shown functionality of WbbO with the aid of *in vitro* assays, *in vivo* galactosyltransferase activity in the presence of galactose have not been presented. The lack of GalT sequences could also have been ascribed to inadequate cDNA library size or underepresented cDNAs in the yeast expression libraries. However, a primary cDNA library titer of 97500 pfu and an amplified 4.16 X 10^{10} pfu was obtained for the expression cDNA library, indicating adequate size. The yeast libraries contained 18000 clones (YEpLac-library) and 12000 clones (pPVD-library), dismissing the possibility of inadequate library size.

While red algae GalT homologues remain elusive, functional complementation of *Gal*E mutants yielded a *Gelidium pristoides* UGE gene. UGE enzymes from agarophytic red seaweed remain largely uncharacterized. To date, a UGE has been purified from freshwater thermophilic micro red algae *Galdieria sulphuraria* (Prosselkov *et al*., 1996), while UGEs

have been enzymatically detected for *Calliblepharis jubata* and *Solieria chordalis* (Goulard *et al*., 1999; Goulard *et al*., 2001; Goulard *et al*., 2003). This is the first study reporting sequence data for red seaweed UGEs. Southern analysis revealed the possibility of two UGE copies in the *Gelidium pristoides* genome, although only a single gene copy was isolated. The *Gelidium pristoides* UGE (*Gp*UGE) gene displays 57 % sequence similarity to known UGEs genes from *E. coli* (BAA35421), *S. cerevisiae* (EDN64634), *H. sapiens* (NP_001121093) and *A. thaliana* (AAC33955). *Gp*UGE protein has conserved domains (GXXGXXG-NAD^+ and Ser and Tyr-X-X-X-Lys motifs) consistent with known UGE enzymes (Figure 2.3.4.). UGE and other epimerases belong to short chain dehydrogenase/reductase (SDR) family and the NADP-Rossmann clan of enzymes (Yin *et al*., 2011). The current hypothesis suggests all 4-epimerases evolved from a common ancestor epimerase, hence their similarity in reaction mechanisms. As a result some plant and bacterial UGE homologoues have shown promiscuity towards different substrates i.e. UDP-GalNAc, UDP-GlcNAc and UDP-D-Xylose (Roper and Ferguson, 2003, Yin *et al*., 2011). Whether or not *Gp*UGE would be able exhibit similar behaviour is unknown. Based on conserved elements it is expected that *Gp*UGE exhibits similar activity to known UGE enzymes i.e. the interconversion of UDP-D-galactose and UDP-D-glucose. However, the low sequence similarity may suggest slight kinetic differences compared to previously described UGEs.

Crude enzyme extracts from *E. coli* PL-2 expressing recombinant *Gp*UGE demonstrated the ability to enzymatically convert UDP-D-galactose to UDP-D-glucose. These results identified *Gp*UGE as a *bona fide* UDP-galactose 4-epimerase. Its activity profile furthermore corresponds with previously described UGE enzymes (Chen *et al*., 1999; Seiboth *et al*., 2002; Roper and Ferguson, 2003; Agarwal *et al*., 2007; Scott and Timson, 2007). Previous experiments based on physiological investigations have suggested UGE involvement in red algae galactan production (Hemmingson *et al*., 1996; Goulard *et al*., 1999; Fournet *et al*., 1999; Goulard *et al*., 2003). However, genetic evidence regarding red algae UGE biochemistry is lacking. It is our intention to further explore this behaviour of *Gelidium pristoides* UGE and functionally characterize this enzyme with respect to galactan biosynthesis in the red algae *Gelidium pristoides*.

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CHAPTER 3

Correlation of activity of the UDP-galactose 4-epimerase with galactan (agar) biosynthesis in Rhodophyte *Gelidium pristoides*

* Chapter 3 in conjunction with Chapter 2 is currently being prepared for publication

3.1. INTRODUCTION

Gelidium pristoides (Turner) Kützing is a Rhodophyte commonly found on the south coast of South Africa. *G.pristoides* is routinely harvested for production of agar for application in food, pharmaceutical and biotechnology (Anderson *et al*., 1991). Given the industrial significance of this cell wall polysaccharide, evidence regarding its biosynthesis is not yet fully understood (Hemmingson *et al*., 1996; Pomin and Mourão, 2008; Genicot-Joncour *et al*., 2009). Stereochemistry dictates that agarans are comprised of alternating residues of D- and L-galactose in α-1,4 and β-1,3 configurations (McCandless and Craigie, 1979; Percival, 1979; Stoddard, 1984; Pomin, 2008**;** Genicot-Joncour *et al*., 2009). Moreover, galactosylation reactions involving these enzymes require UDP-D-galactose as substrate, the product of the Leloir pathway (Hennet, 2002; Berger and Rohrer, 2003). UGE (UDP-galactose 4-epimerase or UDP-glucose 4-epimerase; EC 5.1.3.2), an essential Leloir enzyme, facilitates the freely reversible catalytic conversion between UDP-D-galactose and UDP-D-glucose (Leloir, 1951; Wilson and Hogness, 1964; Frey, 1996). Collectively, UGEs are model enzymes belonging to class II oxidoreductases requiring NAD^+ as a cofactor (Frey, 1996; Holden *et al*., 2003). Previous investigations have demonstrated that liberation of NAD^+ coincides with enzyme denaturation suggesting non-covalent association between UGE catalytic subunits (Frey, 1996, Barat and Bhattacharyya, 2001; Holden *et al*., 2003). However, some epimerases (*A. thaliana* isoform *UGE4)* require exogenous NAD^+ to enhance catalysis (Barber *et al*., 2005).

UGEs have thus far been identified from *E. coli*, *H. sapiens*, bacteria, yeasts and land plants (Dörmann *et al*., 1996; Mozzi *et al*., 2003; Majumdar *et al*., 2004; Nayar *et al*., 2004; Zhang *et al*., 2006; Agarwal *et al*., 2007). Modeling of the epimerase active site has significantly contributed to current knowledge on UGE catalytic mechanisms (Bauer *et al*., 1992; Frey, 1996; Liu *et al*., 1997; Thoden *et al*., 2000; Holden *et al*., 2003). Although organisms expressing UGEs are diverse, the catalytic mechanism is well conserved and can be summarized in three steps: (a) Conformational changes due to binding of UDP-D-galactose cause transfer of the hydride at the C-4 position to the enzyme-bound NAD^+ molecule leading to enzyme-NADH-4-keto-sugar complex formation (b) The 4-keto-sugar intermediate undergoes 180^o rotation, presenting its posterior side to the reduced dinucleotide (c) The hydride from the newly formed NADH is returned to the 4-keto-sugar intermediate leading to restoration and inversion of the C-4 hydroxyl group, producing UDP-D-glucose (Frey, 1996; Holden *et al*., 2003).

This study reports the biochemical characterization of a UGE from an agar producing red seaweed. The gene was one of several UGE clones isolated by functional complementation from a *Gelidium pristoides* cDNA library as discussed in Chapter 2. Its identity was confirmed by functional complementation of a *galE*-deficient *E. coli* mutant. Furthermore, *Gp*UGE was biochemically characterized after heterologous expression in the *E. coli* mutant PL-2. In addition, *in planta* sqPCR investigation of *Gp*UGE transcript levels with respect to dark and light cycling was determined. Our report proposes that the *G. pristoides* UGE (*Gp*UGE) plays a role in agar biosynthesis through provision of UDP-D-galactose.

3.2. MATERIALS AND METHODS

3.2.1. Screening, cloning and expression of *G. pristoides* UGE (*Gp*UGE). *UGE*-deficient *E. coli* mutant PL-2 [Hfr (PO1), *galE28* (GalS), λ^-, *e14-*, *relA1*, *spoT1*, *thi-1*] was used as host for functionally screening a *G. pristoides* expression cDNA library (library construction is described in Chapter 2). Clone 17, a cDNA library clone identified through functional complementation of UGE (Chapter 2), was used as template for PCR amplification of the *G. pristoides* UGE gene (1035 bp). Gene specific primers GpUGE Fwd (5'-CCGGGGATCCAT GACAACAAACAAGACAATTCTCG-3') and GpUGE Rev (5'-GGCCCTCGAGTTATTTA GGACCATAACCATTTGG-3') introduced restriction sites *Bam*HI and *Xho*I, respectively. PCR conditions were as follows; Initial denaturation: 95 ^{o}C for 2 min; Denaturation: 98 ^{o}C for 20 sec; Annealing temperature: 65 ^{o}C for 20 sec; Extension: 72 ^{o}C for 30 sec; Final extension: 72 ^{o}C for 5 min. PCR reactions were performed using KAPA HiFi polymerase (KAPA Biosystems, South Africa) following the manufacturers specifications. Amplicons were cloned into pJET1.2 (Fermentas, USA) to produce pJET-*Gp*UGE. The gene was excised using the PCR incorporated *Bam*HI and *Xho*I restriction sites and directionally cloned to pRSET-A (Invitrogen, Germany).

3.2.2. Heterologous expression of *Gp*UGE in *E. coli* PL-2. A 5 ml starter culture of LB (Luria-Bertani) broth [1 % Tryptone (w/v); 0.5 % Yeast extract (w/v); 0.5 % NaCl (w/v)

supplemented with Ampicillin (50 µg/ ml)] was inoculated with *E. coli* PL-2 cells harbouring pRSET-*Gp*UGE and grown at 37 ^{o}C overnight with shaking. The overnight culture was used to inoculate 200 ml of fresh SOB broth [2 % Tryptone (w/v); 0.5 % Yeast extract (w/v),8.5 mM NaCl; 2.5 mM KCl; 10 mM $MgSO_4$; 10 mM $MgCl_2$; pH 7.0] to OD_{600} = 0.1. Cultures were grown for approximately 4 h until the early-exponential phase (OD_{600} = 0.4 – 0.5) and induced with 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) (Fermentas, USA). Growth was monitored for 6 h after which cells were harvested by centrifugation (5000 *g*, 5 min, 4 ^{o}C) and subjected to protein extraction.

3.2.3. Protein preparation and SDS-PAGE analysis. Crude protein was extracted from *E. coli* PL-2 cultures expressing *Gp*UGE. The gene was harboured by pRSET-A, which allows for His-tag purification of recombinant gene products. Cells were resuspended in 2 ml protein extraction buffer [50 mM Potassium phosphate buffer, pH 7.5; 2 mM DTT; 0.1 % Triton X-100 (v/v); $^{1}/_{2}$ X Protease inhibitor cocktail tablet (Sigma-Aldrich, Germany)] and lysed by three freeze-thaw cycles, followed by three 10 s sonication cycles with intermittent cooling on ice. Insoluble cell debris was removed by centrifugation (16000 *g*, 15 min, 4 ^{o}C) and supernatant transferred to a fresh 1.5 ml eppendorf tubes. For long term storage glycerol was added to a final concentration of 10 % (v/v) and samples were stored at – 80 ^{o}C. Protein concentrations were determined by method of Bradford using 10 µg/ ul Bovine Serum Albumin (BSA) as standard concentration (Bradford *et al*, 1976). To confirm the presence of 3XHis-*Gp*UGE, total protein extract (150 µg) was subjected to separation on an 8 % SDS-polyacrylamide gel (Laemmli, 1970).

3.2.4. Enzyme activity assays. The ability of *Gp*UGE to convert UDP-D-galactose to UDP-D-glucose was coupled to the formation of NADH by UDP-D-glucose dehydrogenase (UGD). Release of NAD^+ was continuously monitored at 340 nm. Standard enzymatic assays (Assay I) unless stated otherwise was conducted with 190 µl Assay buffer I [20 mM Tris-HCl, pH 8.5; 0.25 mM NAD^+; 0.0125 units UPD-glucose dehydrogenase; 2 mM UDP-D-galactose (Sigma-Aldrich, Germany)] and reactions initiated with 100 µg (10 µg/ µl) total protein. Assays for UGE kinetic determinations were performed as discontinuous reactions (Assay II) as described by Wilson and Hogness (Wilson and Hogness, 1964). Assay II was initiated with addition of 10 µl (100 µg total protein) in 90 µl reaction buffer [20 mM Tris-HCl, pH 8.5; 0.25 mM NAD^+; 2 mM UDP-D-galactose (Sigma-Aldrich, Germany)] and reactions

performed for 8 min at 25 °C (Reaction I). Reactions were ceased by immediate immersion in liquid N_2 and boiled at 95 °C for 5 min and assessed for production of UDP-D-glucose. The mixture from Reaction 1 (100 µl) was added to 100 µl Assay buffer II [20 mM Tris-HCl, pH 8.5; 0.25 mM NAD^+; 0.0125 units of UGD (Sigma-Aldrich, Germany)] and the formation of NADH was continuously monitored at 340 nm. For standardization: one unit of epimerase activity is defined as 1 µmol of UDP-D-glucose produced per min. Oxidation of 1 mol UDP-D-glucose produces 2 mol NADH. Assays represent means ± SE of 6 replicates.

3.2.5. Determination of pH optima for *G. pristoides* UGE. pH optimum was determined by measuring UGE activity in various 50 mM buffers ranging from pH 5.0 to pH 10.0 (Full strength McIlvaine's buffer, pH 5.0 to pH 6.0; MES-KOH, pH 6.0 to pH 7.0; HEPES-KOH, pH 7.0 to pH 9.0; Sodium carbonate buffer, pH 9.0 to pH 10.0). Protein samples were desalted with Sephadex G-25 Fine (G.E. Healthcare, USA) columns in a final bed volume of 3 ml to facilitate buffer exchange. Epimerase assays were conducted subsequent to buffer exchange. Activity was measured as discontinuous reactions (Assay II) as described in previous section.

3.2.6. Determination of temperature optimum for *Gp*UGE. Epimerase activity was investigated at temperatures ranging from 0 °C to 90 °C. Assays were performed in reaction buffer [20 mM Tris-HCl, pH 8.5; 2 mM UDP-D-galactose (Sigma-Aldrich, Germany)] and performed for 8 min at different temperatures (0 °C to 90 °C). Reactions were terminated by immersion in liquid N_2 and boiled at 95 °C for 5 min. UDP-D-glucose content was subsequently measured with Assay buffer II in a total volume of 200 µl.

3.2.7. Kinetics of *Gp*UGE. Michaelis-Menten kinetics for the epimerase reverse reaction was assessed with increasing concentrations of UDP-D-galactose from 0 – 8 mM using reaction conditions for Assay II. *Gp*UGE kinetic data was analyzed using Prism 5.0 (Graphpad, USA). Determination of kinetic parameters K_m and V_{max} was calculated using Michaelis-Menten (non-linear regression) plots. The turnover number (k_{cat}) was calculated using Prism 5.0 (Graphpad, USA) using the following equation: $k_{cat} = V_{max}$ / Molecular concentration of the enzyme.

3.2.8. RNA isolations and semi-quantitative RT-PCR. Actively growing *G. pristoides* plants were collected from wild populations (Kalk Bay, Cape Town, South Africa) at low tide during the day as well as at night. Total RNA was extracted as previously described (Valderrama-Cháirez *et al*., 2002) with slight modifications as outlined in Chapter 2. Two micrograms of total RNA was converted to single-stranded cDNA (RevertAid First Strand cDNA synthesis kit, Fermentas, USA) and diluted to 250 ng/ul prior to RT-PCR analysis. PCR reactions were performed with 2 µl (500 ng) cDNA. RT-PCR analysis was conducted with constitutively expressed 18S rRNA and *RuBisCo* (Ribulose-1,5-bisphosphate carboxylase/ oxygenase) genes used as controls. For RT-PCR analysis of 18S rRNA gene, Gp18S Fwd (5'-GGCGTTTGCCTTGTACACATTAGC-3') and Gp18S Rev (5'-GGCTGTC AATCCTCACTATGTCCG-3') were used as primers. To assess constitutive expression of *RuBisCo* gene, primers GpRuBisCo Fwd (5'-GGTTCTATTGCTGCGACAGGC-3') and GpRuBisCo Rev (5'-CCGGATACCACCAGATGCTACT GG-3') were used. *Gp*UGE expression was assessed with primers GpUGE_Sq Fwd (5'-CCGCTCCAATTTCAACATTC TCAGTTACTG-3') and GpUGE_Sq Rev (5'-GGATGACAACAAACAAGACAATTCTCG-3'). RT-PCR analysis conditions were as follows; Initial denaturation: 95 ^{o}C for 1 min; Denaturation: 95 ^{o}C for 30 sec; Annealing temperature: 55 ^{o}C for 30 sec; Extension: 72 ^{o}C for 30 sec; Final extension: 72 ^{o}C for 5 min. PCR was performed with MyTaq polymerase (Bioline, USA) for 27 cycles according to manufacturer's instructions.

3.3. RESULTS

3.3.1. *Gelidium pristoides* cDNA library complements *E. coli* PL-2 mutant

A single clone (Clone 17) was selected for further study (See Chapter 2) based on its ability to restore galactose metabolism in *E. coli* PL-2 mutant cells. BLASTX analysis of Clone 17 DNA sequence revealed 65 % similarity to a UDP-galactose 4-epimerase (UGE) gene from *Phaeodactylum tricornutum* (XP_002185705.1). Amino acid sequence comparisons of *Gelidium pristoides* UGE (*Gp*UGE) to previously characterized UGE enzymes indicated an

overall identity of 55 % (Figure 3.3.1.). Alignments indicated conserved regions (GXXGXXG-NAD^+ binding motifs and Ser and Tyr-X-X-X-Lys amino acid residues) consistent with characterized UGE enzymes. Hydrophobicity plots (performed according to TMPred server http://www.ch.embnet.org/software/TMPRED_form.html) suggested 3 membrane-spanning regions (Figure 3.3.2.), suggesting *Gp*UGE membrane association.

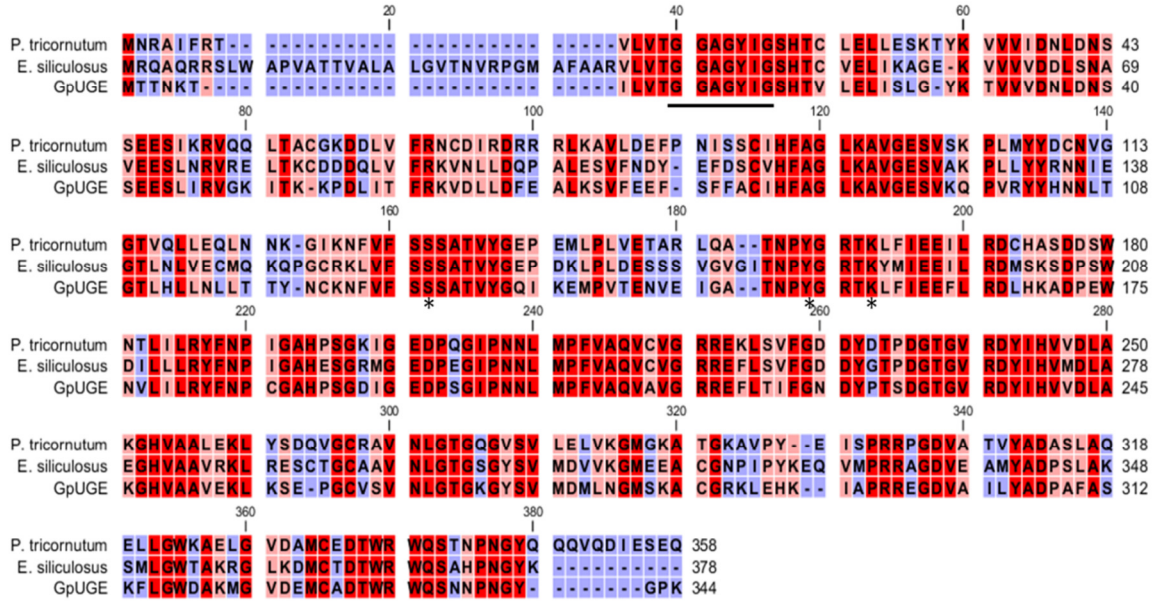


Figure 3.3.1. Amino acid sequence comparison of *Gp*UGE against UGEs from marine organisms. GenBank accession numbers; *P. tricornutum* (ACI65175.1) and *E. siliculosus* (CBJ29580.1). Amino acid residues highlighted in red indicate conservancy. Underlined residues indicate the conserved NAD binding motif GXXGXXG. The conserved Ser and Tyr-X-X-X-Lys residues are each marked by asterisks. Multiple alignments were performed with CLC Genomics Workbench.

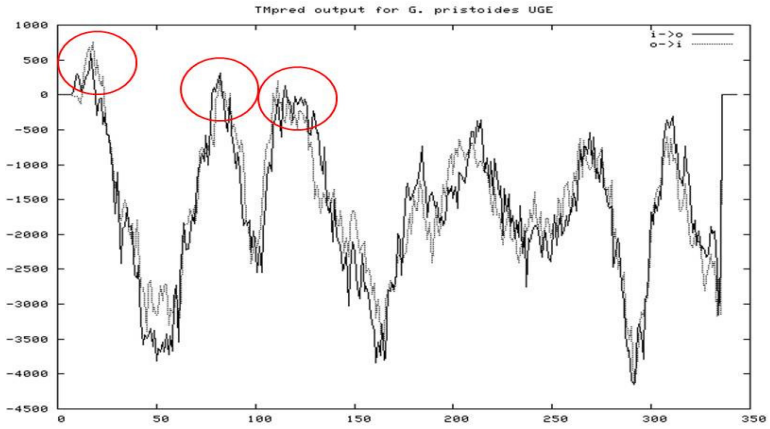


Figure 3.3.2. Prediction of possible membrane spanning domains of *Gp*UGE. *Gp*UGE N-terminus contains 3 trans-membrane domains predicted by TMPred server http://www.ch.embnet.org/software/TMPRED_form.html.

3.3.2. Expression of *Gp*UGE in *E. coli* PL-2

The *Gp*UGE gene was PCR amplified and cloned into the inducible expression vector pRSET-A (Figure 3.3.3.). Plate assays suggested that Clone 17 and *E. coli* PL-2 cells carrying pRSET-*Gp*UGE displayed increased ability to survive on galactose in contrast to galactose sensitive mutant cells (*E. coli* PL-2). SDS-PAGE analysis of mutant *E. coli* expressing pRSET-*Gp*UGE showed increased levels of recombinant *Gp*UGE production with respect to the control (untransformed *E. coli* PL-2) over a 6 hour growth period. SDS-PAGE analysis suggested a molecular size of 41 kDa which includes the 3XHis-tag (Figure 3.3.4.).

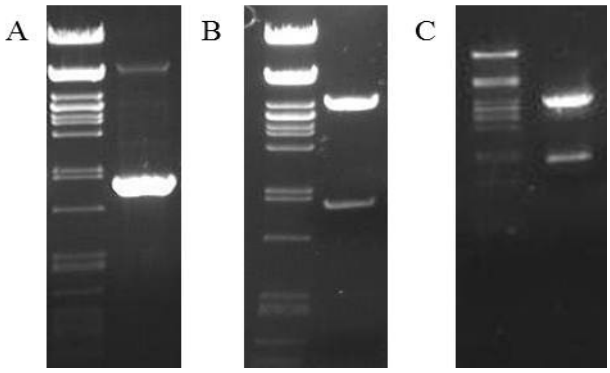


Figure 3.3.3. Cloning of *Gelidium pristoides* UGE (*Gp*UGE). (A) *Gp*UGE was PCR amplified from Clone 17 and cloned into pJET (Fermentas, USA) (B) Restriction digestion of pJET-*Gp*UGE and cloned into pRSET-A (*Bam*HI/*Xho*I) (C) pRSET-*Gp*UGE restriction digested with *Bam*HI and *Xho*I.

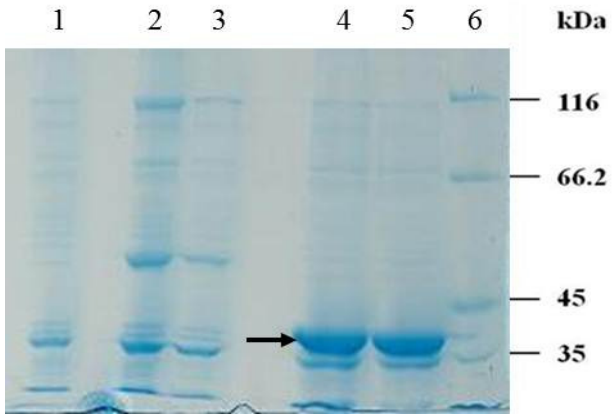


Figure 3.3.4. SDS-PAGE analysis of overexpressed 6XHis-*Gp*UGE. Expression was induced with 1 mM IPTG in *E. coli* PL-2 cultures harboring pRSET-*Gp*UGE and control cells grown for 4 hours. Crude protein prepared and 150 µg protein separated on 8 % (v/v) SDS-PAGE gel. Lane 1: empty pRSETA control. Lane 2 and 3: pRSETA with a control gene. Lane 3 and 4: Overexpressed pRSET-*Gp*UGE. Lane 6: Protein molecular weight marker in kDa.

3.3.3. Catalytic properties of *G. pristoides UGE*

Crude extracts from *Gp*UGE overexpressed in *E. coli* PL-2 mutant background displayed marked increased activity in contrast to crude extracts from untransformed mutant cells under standard assay conditions (See Figure 2.3.6). As expected, a linear correlation was obtained between increased epimerase activity and increased protein concentrations under standard assay conditions (Figure 3.3.5.). We also observed increased *Gp*UGE activity when NAD^+ was exogenously supplied (Figure 3.3.6.).

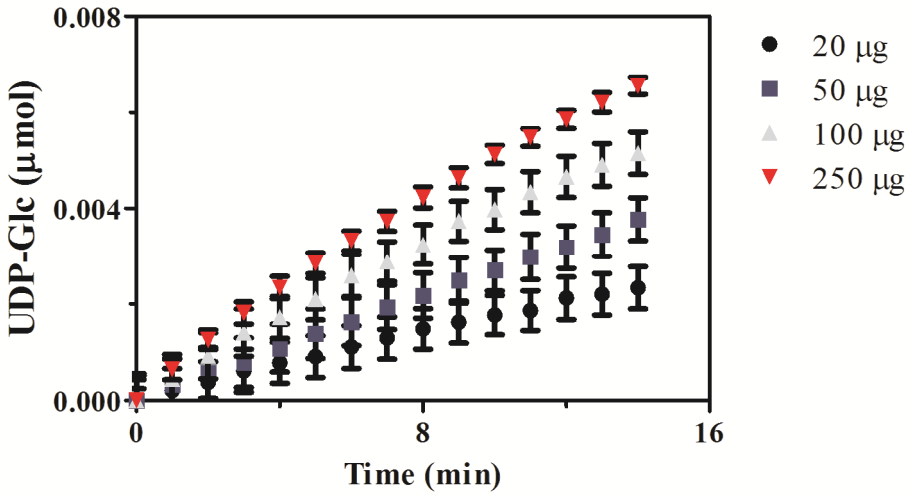


Figure 3.3.5. Increasing epimerase activity of *Gp*UGE. The effect of increased *Gp*UGE enzyme concentration was monitored under standard conditions described for assay I. Activity was monitored over 15 min by incrementally increasing enzyme concentration from 20 ug to 250 ug. A linear correlation could be established between increased enzyme concentration and increased formation of UDP-D-glucose. Black circle 20 µg (●), purple square (■) 50 µg, silver triangle (▲) 100 µg, red triangle (▼) 250 µg protein. Presented data represents ± S.E.M. of 6 independent replicates.

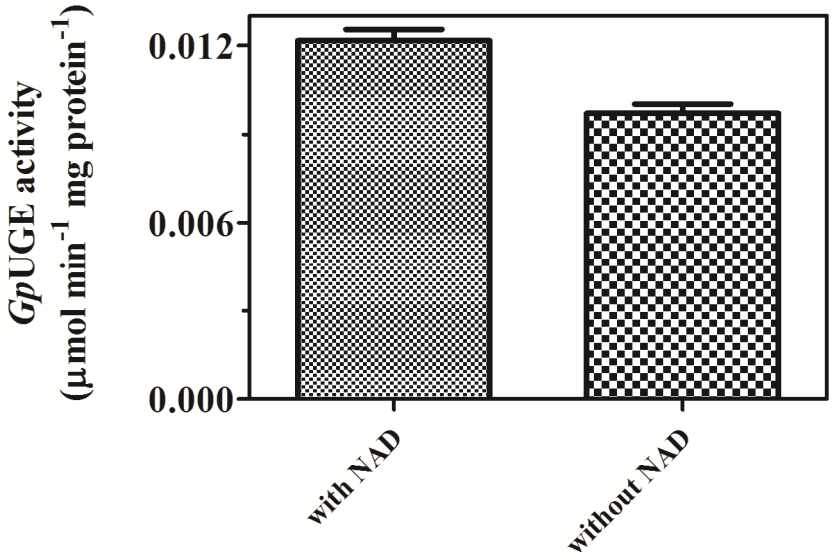


Figure 3.3.6. Recombinant 3XHis*Gp*UGE dependence on NAD^+. Reactions were performed for 8 min after which accumulated UDP-D-glucose content was measured via UDP-D-glucose dehydrogenase (Assay II). (A) Reactions with exogenously added NAD^+ and (B) without NAD^+. Each data point represents ± S.E.M. of 3 independent replicates

3.3.4. pH and temperature optimum of *G. pristoides* UGE

Epimerase assays were conducted in various buffers ranging from pH 5 to 10. Significant *Gp*UGE activity was measured between pH 6.5 and 10.0 with optimum activity between pH 8 and 9 (Figure 3.3.7.A.). Enzyme activity was also assessed at different temperatures (0 – 90 ^oC). The *Gp*UGE functioned optimally at mesophillic temperatures (25 to 30 ^oC) and remained stable during the reaction time of 8 minutes from 10 – 40 ^oC (Figure 3.3.7.B.).

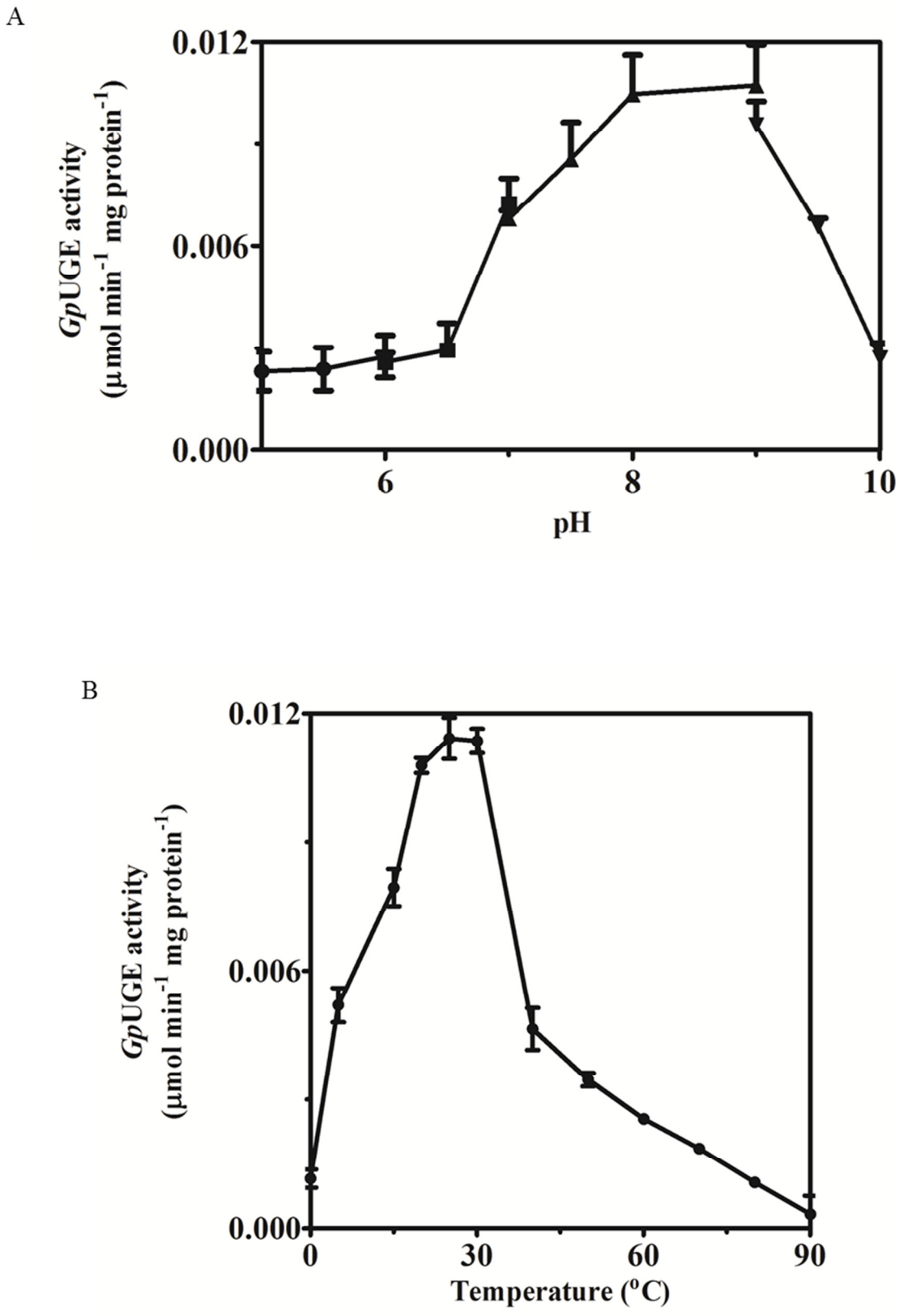
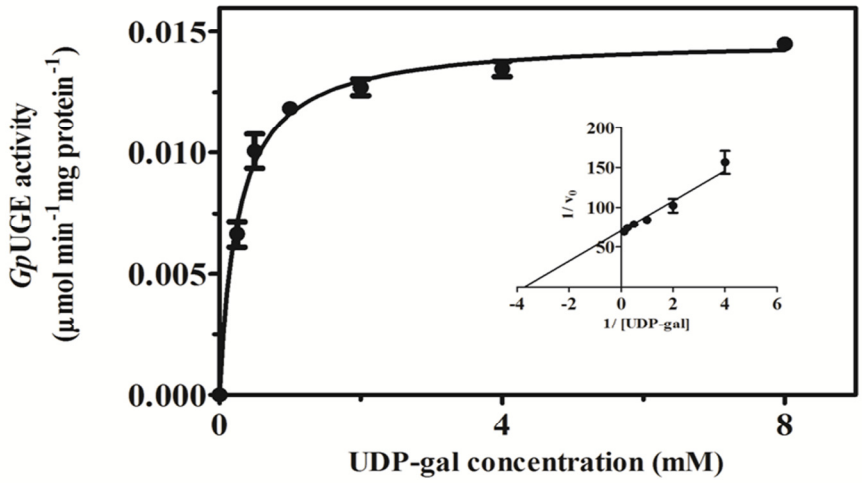


Figure 3.3.7. Effect of pH and temperature on recombinant 3XHis*Gp*UGE activity. (A) UGE activity was assessed in different buffers ranging from pH 5 to 10. (B) Thermal stability was determined after 8 min at temperatures ranging from 0 °C to 90 °C. Data presented in both experimental sets represent ± S.E.M of 6 independent replicates.

3.3.5. Kinetic properties of *G. pristoides* UGE

Kinetics for the *Gp*UGE catalytic reaction was determined in the presence of increasing amounts of substrate (0 – 8 mM UDP-D-galactose). *Gp*UGE exhibited classical Michaelis-Menten kinetics under standard experimental conditions (25 ^{o}C and pH 8.5) with an average activity of 30 µmol/min/mg protein. The observed K_m for *Gp*UGE is 0.27 mM and V_{max} = 0.015 µmol/min/mg protein (Figure 3.3.8.A). We calculated turnover number (k_{cat}) for the crude enzyme extract to be 1206 s^{-1}. The metabolite 5'-UMP was shown to be a strict competitive inhibitor of epimerase activity *in vitro* (Kalckar *et al*., 1970; Majumdar *et al*., 2004; Nayar *et al*., 2004; Brahma *et al*., 2009). *Gp*UGE kinetics was conducted in the presence increasing inhibitor concentrations ranging from 0 to 16 mM (Figure 3.3.8.B.). Increasing 5'-UMP concentrations drastically reduced epimerase activity. However, this inhibitor did not completely abolish enzyme activity. 5'-UMP and UDP-D-galactose simultaneously compete for binding to the enzyme catalytic site, resulting in reduced action at increasing concentrations.

A



B

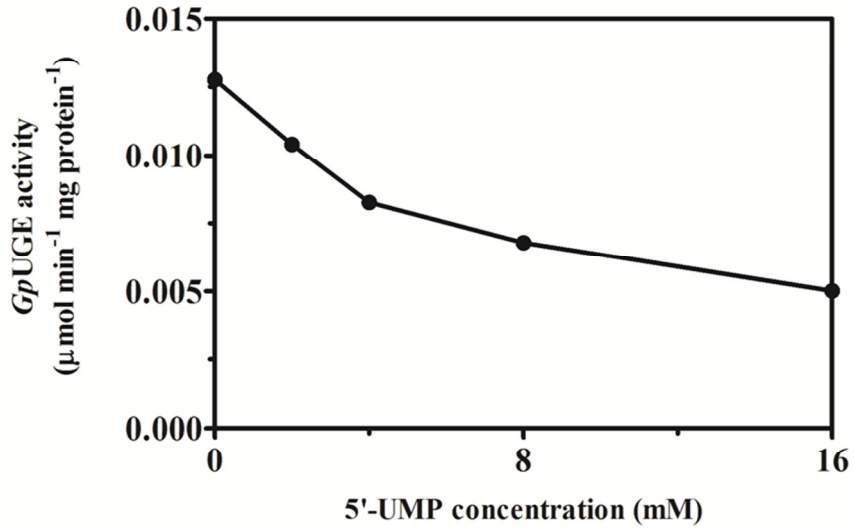


Figure 3.3.8. Kinetic analysis of recombinant 3XHis*Gp*UGE. (A) Michaelis-Menten plot (v_0 versus [s]) indicating K_m and V_{max} of *Gp*UGE. Enzyme activity was performed using UDP-D-galactose at different concentrations ranging from 0 to 8 mM. K_m = 0.27 mM and V_{max} = 0.015 µmol/min/mg. k_{cat} for *Gp*UGE catalyzed reaction is 1206 s^{-1}. Inset: Lineweaver-Burk plot (B) Inhibition of *Gp*UGE by 5'-UMP. Epimerase inhibition was monitored in the presence of increasing 5'-UMP (0 to 16 mM) at constant UDP-D-galactose concentration (2 mM). Reaction conditions were performed as per assay II as stated in the Materials and Methods. Each data point in both experimental sets represents ± S.E.M. of 6 independent replicates.

3.3.7. *G. pristoides* UGE expression profiles during light and dark

To investigate the involvement of *Gp*UGE in agar production, day and night transcript profiles was monitored by semi-quantitative RT-PCR analysis. PCR-derived *Gp*UGE amplicons were compared to constitutively expressed *RuBisCo* (Ribulose-1,5-bisphosphate carboxylase/ oxygenase) and 18S rRNA gene transcripts (Figure 3.3.9.). RT-PCR analysis showed higher nocturnal *Gp*UGE expression levels, in contrast to lower levels exhibited by light exposed plants. In both light and dark phases *RuBisCo* and 18S rRNA gene expression was maintained at normal levels in relation to fluctuating *Gp*UGE transcripts.

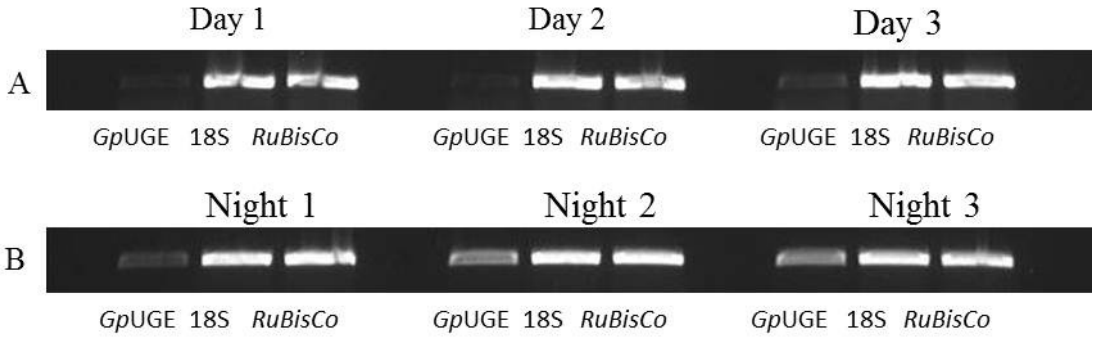


Figure 3.3.9. Investigation of *Gp*UGE transcript profile of light and dark exposed whole *G. pristoides* plants. Total RNA extracted from *G. pristoides* plants was subjected to semi-quantitative RT-PCR analysis. PCR-generated *Gp*UGE amplicons were compared to constitutively expressed 18S RNA and *RuBisCo* transcripts. (A) *Gp*UGE expression profile in plants collected at midday versus transcript levels during the nocturnal cycle (22h00) (B). PCR amplifications were performed for 27 cycles until saturation was achieved. RT analyses were performed for 3 biological replicates for each represented gene.

3.4. DISCUSSION

Epimerases are found in all living cells and their catalytic abilities are essential for many structural and metabolic functions. These enzymes are mainly known for their nucleotide sugar interconversion abilities, and several epimerization mechanisms have evolved to

accommodate the diversity of activated sugars (Reiter and Vanzin, 2001; Seifert, 2004; Rösti *et al*., 2007, Yin *et al*., 2011). In plants epimerases alongside monosaccharide kinases and NDP-sugar pyrophosphorylases also synthesize nucleotide sugars as part of the various monosaccharide salvage pathways. However, their primary function is the synthesis of nucleotide sugars via manipulation of the sugar moiety in UDP-D-glucose or GDP-D-mannose mainly for production of UDP-D-galactose, GDP-L-galactose and GDP-L-fucose (Reiter and Vanzin, 2001). The conversion of nucleotide sugars (UDP-D-glucose and GDP-D-mannose) to GDP-L-galactose and UDP-D-galactose require the action of GDP-mannose 3,5-epimerase and UDP-galactose 4-epimerase, respectively.

In recent years, UDP-galactose 4-epimerase (UGE) has received significant attention due to its interesting catalytic mechanism by which UDP-D-galactose is converted to UDP-D-glucose and vice versa. Its role in the Leloir pathway is also well documented (Frey, 1996; Holden *et al*., 2003). Several reports from green plants and bacteria have furthermore implicated UGE in cell wall polysaccharide biosynthesis (Joersbo *et al*., 1999; Degeest and De Vuyst, 2000; Mozzi *et al*., 2003; Barber *et al*., 2005; Rösti *et al*., 2007). The goal of this study was to characterize a UGE from an agar-accumulating macro red alga (*Gelidium pristoides*) and investigate its involvement in agar biosynthesis. While the enzyme has been characterized from fresh water thermophilic red algae (*Galdieria sulphuraria)*, this study reports the biochemical characterization of a UGE from a marine red seaweed.

*Gp*UGE has been identified through complementation of the *E. coli* PL-2 UGE mutant (Chaper 2). The gene was PCR amplified and enzyme activity was confirmed by heterologous expression in an *E. coli* mutant. The enzyme also displayed activity in *E. coli* crude enzyme extracts in the presence of UDP-D-galactose (Figure 2.3.8. and Figure 3.3.4.). Recombinant *Gp*UGE exhibited markedly increased activity compared to untransformed mutant cells. *Gp*UGE was subsequently characterized and the biochemical parameters for *Gp*UGE determined under standard experimental conditions (25 ^{o}C and pH 8.5). The enzyme exhibited a average activity of 30 µmol/min/mg protein, similar to previous findings in *S. cerevisiae* (31.8 µmol/min/mg protein), *E. coli* (27 µmol/min/mg protein), *P. sativum* (38.9 µmol/min/mg protein) and micro red algae *Galdieria sulphuraria* (42 µmol/min/mg protein) (Fukasawa *et al*., 1982; Prosselkov *et al*., 1996; Chen *et al*., 1999; Kotake *et al*., 2009). The *Gp*UGE enzyme exhibited classic Michaelis-Menten kinetics with an observed K_m of 0.27 mM and V_{max} = 0.015 µmol/min/mg protein (Figure 3.3.7.A). The K_m closely resembles that

of golden algae *Poterioochromonas malhamensis* (0.26 mM), but differs from micro red algae *Galdiera sulphuraria* (0.064 mM) (Thomson *et al*., 1984; Prosselkov *et al*., 1996; Brahma *et al*., 2009). The catalytic turnover number (k_{cat}) for the crude enzyme extract was calculated to be 1206 s^{-1}.

Catalytic behavior of *Gp*UGE was investigated at varying pH and temperatures. The epimerase functioned optimally between pH 8.0 and 9.0, similar to other UGEs (Wilson and Hogness, 1964; Dey, 1984; Chen *et al*., 1999; Berger *et al*., 2001; Agarwal *et al*., 2007; Nayar *et al*., 2004). Enzyme activity was optimal at mesophillic temperatures between 25 ^{o}C and 30 ^{o}C. This was also reflected by similar values obtained for other UGEs.

*Gp*UGE *in vitro* assays showed enhanced performance in the presence of exogenously added NAD^+. Similar observations were reported for *A. thaliana* isoforms *AtUGE2* and *AtUGE4* (Barber *et al*., 2005). Unlike *A. thaliana* isoforms, *Gp*UGE activity retained activity in the absence of added NAD^+. *AtUGE4* has been indicated to have possible Golgi membrane association, in contrast to the cytosolic location of UGE isoforms unaffected by the co-factor (Barber *et al*., 2005). It was speculated that Golgi-associated UGEs in *A. thaliana* depend on fluctuations in the redox state of NAD^+ thereby regulating galactose availability for cell wall incorporation (Seifert *et al*., 2002; Seifert, 2004; Barber *et al*., 2005; Rösti *et al*., 2007). Three possible membrane spanning regions were identified in *Gp*UGE, supporting claims of Golgi membrane localization. Whether this is indeed the scenario in *G. pristoides* remains to be investigated.

Various UGE isoforms were found to have roles in cell wall polysaccharide biosynthesis in terrestrial plants. *A. thaliana UGE2* and *UGE4* mutant lines showed adverse effects in cell wall galactose content (Barber *et al*., 2005). In red algae evidence suggests links between UGE activity and agar biosynthesis. Light deprived *Gracilaria* spp. incubated with C^{13}-glucose, was shown to incorporate the radiolabel into galactan (Hemmingson *et al*., 1996a; Hemmingson *et al*., 1996b). Identical results were demonstrated for ascidians (Mourão, 1990). These results are suggestive of UGE activity as the incorporation of radio-label into galactan can only be explained via an epimerization event from glucose to galactose. UGE activity was also measured in crude extracts of red algae where it appears to be involved in

the production of UDP-D-galactose for polysaccharide synthesis (Prosselkov *et al*., 1996; Goulard *et al*., 1999; Goulard *et al*., 2003). Significant UGE activity has furthermore been detected in galactomannan accumulating guar seeds (Joersbo *et al*., 1999). The authors directly implicated the UGE enzyme for supplying UDP-D-galactose for production of this galactose-containing polysaccharide. Given the possible involvement of UGE in galactan synthesis, this study investigated *in vivo* *Gp*UGE expression in wild populations of *G. pristoides*. Semi-quantitative RT-PCR analysis of day and night collected algae indicated higher UGE transcript levels in plants exposed to darkness. Higher UGE expression levels in *A. thaliana* were linked to the depletion of UDP-D-galactose reserves destined for cell wall polysaccharide biosynthesis, suggesting that UGEs have a higher affinity for UDP-D-galactose (Dörmann and Benning, 1998). Such a scenario is unlikely in red algae, since production of UDP-D-galactose (by UGE) is shifted towards accumulation of the nucleotide sugar (Fournet *et al*., 1999; Goulard *et al*., 1999). UDP-D-galactose reserves in red algae *S. chordalis* and *C. jubata* were reported to be in excess in contrast to diminished levels of all other nucleotide sugars (Goulard *et al*., 1999; Goulard *et al*., 2003). This discovery made by Goulard and co-workers in red algae, *S. chordalis* and *C. jubata*, supports our theory of UDP-D-galactose accumulation in *G. pristoides* as a result of UGE activity. It is speculated that UGE is required to provide UDP-D-galactose during day and night cycles for sulfated galactan and floridoside production, respectively (Macler, 1986; Hemmingson *et al*., 1996; Fournet *et al*., 1999). It was noticed in green tissues (*A. thaliana* and *Solanum tuberosum*) that UGEs have multiple physiological roles (Oomen *et al*., 2004; Barber *et al*., 2005; Zhang *et al*., 2006; Rösti *et al*., 2007). Whether or not this scenario is reflected in *G. pristoides* is not known. Our evidence points to two *Gp*UGE gene copies in the genome of *G. pristoides* (inferred from Chapter 2). The primary role of UGE in *G. pristoides* is in the Leloir pathway i.e. UDP-D-galactose provision for cellular processes such as cell wall polysaccharide biosynthesis (Frey, 1996; Holden *et al*., 2003). Secondly, its involvement in the biosynthesis of secondary storage polysaccharide floridoside (2-*O*-glycerol-*α*-D-galactose) has been documented (Macler, 1986; Hemmingson *et al*., 1996; Prosselkov *et al*., 1996). It has furthermore been suggested that UGE is involved in galactose salvaging and recycling (Reiter and Vanzin, 2001). Considering its different roles in cellular processes, UGE is likely to be ubiquitously expressed in all tissue types, maintaining cellular UDP-D-galactose levels during night and day. We found increased *Gp*UGE expression levels during the night cycle which correlates favourably with nocturnal floridean starch metabolism. It is known that floridean starch is metabolized to UDP-D-glucose during respiration. In addition, previous findings suggest correlation between floridean starch metabolism and agar biosynthesis (Macler, 1986;

Hemmingson *et al*., 1996; Fournet *et al*., 1999). Accumulated evidence suggests a possible relationship between elevated nocturnal UGE activity and agar biosynthesis. The most likely explanation for this correlation is the coordination between respiration and floridean starch metabolism, which causes a rise UGE expression levels. Consequently, the resulting abundant UDP-D-glucose is converted to UDP-D-galactose via *Gp*UGE to possibly supply demand for agar production in addition to other nocturnal activities. UGE expression during the light phase was found to be diminished. Results suggest demand for UDP-D-galactose during the day may be minimal due photosynthesis. Floridoside biosynthesis also occurs in daylight and it is likely that low UGE expression levels are assisting in this process. In the context of *E. coli* expression, the apparent biochemical parameters of recombinant *Gp*UGE coincide with that of other species (some algae UGEs). It was previously shown that UGE activity is linked to cell wall polysaccharide synthesis in various species (Hemmingson *et al*., 1996a; Prosselkov *et al*., 1996; Dörmann and Benning, 1998; Goulard *et al*., 1999; Joersbo *et al*., 1999; Goulard *et al*., 2001; Seifert *et al*., 2002; Goulard *et al*., 2003; Mozzi *et al*., 2003; Barber *et al*., 2005; Rösti *et al*., 2007; Singh *et al*., 2007; El-Ganiny *et al*., 2010). Indeed our sqPCR analysis shows increased nocturnal *Gp*UGE expression which coincides with floridean starch metabolism and possible cell wall polysaccharide biosynthesis through production of UDP-D-galactose.

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CHAPTER 4

De novo sequencing of *Gelidium pristoides* transcriptome

4.1. INTRODUCTION

Sulfated polysaccharides produced by red algae (agar, agarose and carrageenan) are of commercial importance due to their application in food industries adding texture and rheology to food stuffs (Radmer, 1996; De Ruiter and Rudolph, 1997; Pomin and Mourão, 2008; Reddy *et al*., 2010). These polymers are furthermore potential pharmaceutical therapeutics as anti-coagulant, anti-viral and anti-inflammatory agents (Farias *et al*., 2000; Pereira *et al*., 2005). Agar and its derivative agarose, collectively known as agarans, have high value applications in the molecular biotechnology industry (Radmer, 1996). Agarans and carrageenans consist of linear repetitive galactose residues and are classified as homopolysaccharides. Stereochemistry dictate that they consist of alternating α-1,4- and β-1,3-galactose residues, implying the action of α- and β-galactosyltransferases (Percival, 1979; McCandless, 1979; Genicjour *et al*., 2009). Sulfated galactans also possess minor structural modifications to the galactose backbone in the form of sulfate esters (Lahaye, 2001). These groups have fixed carbon positions which are organism specific and require the action of specific sulfotransferase enzymes (Rees, 1961a; Rees 1961b; Hemmingson *et al*., 1996; Lahaye, 2001). Sulfated galactose monomers are further modified by sulfurylases to form of 3,6-anhydro-galactose residues (Lahaye, 2001; Genicjour *et al*., 2009). These residues promote favorable interaction with water molecules. Hence commercial availability of galactans with high 3,6-anhydro-galactose content. Although, red algae (Rhodophyta) have an extended lineage of investigation, many of their metabolic and cellular processes are unknown (Collén *et al*., 2006). Similarly, pathways for sulfated galactan production still remain obscure (Collén *et al*., 2006; Genicjour *et al*., 2009). Many attempts at identifying sulfated galactan biosynthesis-associated genes have been unsuccessful (Lluisma and Ragan, 1997; Lee *et al*., 2000; Weber *et al*., 2004; Collén *et al*., 2006; Lee *et al*., 2007). These efforts relied heavily on expressed sequence tag (EST) approaches with analyses being hampered by limited availability of quality genomic data for red seaweed. The only complete red algae genomes available on NCBI are those of *Guillardia theta* and *Cyanidioschyzon merolae* (Douglas *et al*., 2001; Matsuzaki *et al*., 2004). Due to a lack of reference sequence information annotation of red algae sequences remains a challenge (Lluisma and Ragan, 1997; Weber *et al*., 2004; Grossman, 2005; Lee *et al*., 2007).

New developments in high throughput sequencing coupled with *de novo* assembly facilitate transcriptome data processing for organisms lacking in sequence data (Rismani-Yazdi *et al*.,

2011). Conventional automated Sanger method sequencing relied heavily on steps prior to DNA cloning for the creation of large libraries which served as template for sequencing reactions (Sanger *et al*., 1977). These techniques required extended laboratory practices, while application was limited, offering few downstream advantages for the user. So called "Next generation sequencing" relies on a combination of template preparation, sequence imaging and assembly (Ansorge, 2009; Metzker, 2010). These developments permits direct sequencing from amplified DNA fragments allowing for sequencing projects to be completed in less time and the generation of vast quantities of information. Drawbacks include short sequence reads and computer software limitations (Mardis, 2008; Ansorge, 2009; Metzker, 2010; Paszkiewicz and Studholme, 2010). Ultra-high throughput is set to revolutionize molecular biology as the preferred method of transcriptome analysis making currentmicroarray technologies obsolete (Metzker, 2010; Paszkiewicz and Studholme, 2010). Already significant advances have been made with the announcement of the 1000 genomes project and transcriptome sequencing of various organisms including some green algae species (Emrich 2007; Szittya *et al*., 2008; Bateman and Quackenbush, 2009; Guo *et al*., 2010; Rismani-Yazdi *et al*., 2011).

Gelidium pristoides is a prolific agar producing red algae species of commercial and academic interest (Anderson *et al*., 1991). Lack of sequence information for *G. pristoides* is reflected by GenBank searches yielding only three entries. Given recent successes in Next generation sequencing, the main objective of this study was to sequence the transcriptome of a local *G. pristoides* species in an attempt to decipher agar biosynthesis.

4.2. MATERIALS AND METHODS

4.2.1. Transcriptome sequencing and *de novo* assembly of *G. pristoides* sequence data. *Gelidium pristoides* RNA was extracted as described previously (See Chapter 2) (Valderrama-Cháirez *et al*., 2002). mRNA was separated from total RNA population using GenElute™ mRNA Miniprep system (Sigma-Aldrich, Germany). The nucleic acid was

concentrated by overnight precipitation with $^{1}/_{10}$ volume RNase-free 3 M Sodium acetate ($CH_3COOHNa$), 1 µl (20 µg) Glycogen (Invitrogen, Germany) and 3 volumes absolute ethanol at -20 ^{o}C. Precipitated mRNA was collected by centrifugation (16000 *g*, 40 min, 4 ^{o}C) and washed with 70 % ethanol (v/v). The ethanol was gently removed by pipetting and residual ethanol aspirated in a laminar flow cabinet. Dried mRNA was resuspended in 10 µl RNase-free DEPC H_2O and subsequently converted to double stranded cDNA (RevertAid^{TM} cDNA Synthesis Kit, Fermentas, USA) with slight modifications. For synthesis of first strand cDNA standard oligo-dT primer was replaced with oligo-dT(*Not*I) (5'-CCGGGCGGCCGCTTTTTTTTTTTTTTTTTTTVN-3') containing a *Not*I restriction site (Jarvie and Harkins, 2008). The rest of the procedure was executed as per manufacturers instructions. In total of 4.4 µg (140 µl) of cDNA was subjected to high throughput sequencing (Roche 454 FLX Genome Sequencer system, Inqaba Biotechnologies, South Africa). Sequence processing and contig assembly was performed using Newbler software version 2.3 (Roche Life Sciences, USA).

4.2.2. Sequence annotation. Generated sequence data was analyzed using National Center for Bioinformatics Institute (NCBI) BLASTX alogorithm (Altshul *et al*., 1990). In order to create more complexity, multiple BLAST queries were performed to different databases to identify potential genes of interest. BLASTX searches were performed in the following manner: queries were alternatively conducted against NCBI non-redundant (NR) and Uni Prot/ Swiss-Prot databases against Bacterial taxid sequences. Alternatively, contigs sequences were aligned against Viridiplantae NR and Uni Prot/ Swiss-Prot protein databases following identical procedures.

4.3. RESULTS

4.3.1. Transcriptome sequencing, *de novo* assembly and general characteristics of *Gelidium pristoides* contigs

Total cDNA synthesized from mRNA extracted from intertidal red seaweed *G. pristoides* was subjected to high throughput sequencing using Roche 454 FLX platform. In total, 4.4 µg

cDNA was sequenced which generated approximately 4 Mbp data. A summary of the sequencing and assembly reports are presented in Table 4.3.1. Overall, 18266 raw reads were obtained of which 18207 were assembled by Inqaba Biotechnologies using Newbler software v.2.3 (Roche 454 Life Sciences, USA). Trimming and filtering of low complexity and low quality scores resulted in 5432 reads assembled into 371 contiguous sequences (contigs). Contig sizes ranged from 26 to 2647 bp with an average sequence length of 688 bp (Figure 4.3.1.). Assembly generated 338 isocontigs assembled with an average size of 562 bp (Table 4.3.1.).

Table 4.3.1. Summary of contig assembly report as generated by Inqaba Biotechnologies using Newbler software v2.3.

	Sequencing
Sequencing	
Raw sequencing reads	18266
Reads used	18207
Pre-assembly	
Reads in assembly	7164
Assembly	
Reads assembled as contigs	5432
Isotigs	
Number of isotigs	338
Average length of isotigs	562
Contigs	
Range of contig length	26 – 2647
Average size of contigs	688

4.3.2. Annotation overview of *G. pristoides* contigs

All 371 contigs generated from approx. 4 Mbp data were aligned against eukaryotic proteins posted in the National Center for Bioinformatics Institute (NCBI) sequence databases using the BLASTX alogorithm (Altshul *et al*., 1990). Defined metabolic profiles were created for each BLAST query and these were categorized according to physiological groups (Figure 4.3.2.). Based on the NCBI Non-redundant (NR) database, approximately 69 % of contigs displayed sequence homology with eukaryotic proteins (37 % known and 32 % hypothetical) (Figure 4.3.2.A.). Only 12.4 % of the transcripts were homologous to known red algae sequences obtained from *Pyropia yezoensis*, *Gracilaria gracilis*, *Guillardia theta*, *Griffithsia japonica*, *Chondrus crispus* and *Galdieria sulphuraria*. Low alignment frequencies are attributed to a general shortage of red algae sequence data available on NCBI. In comparison, analysis performed utilizing the NCBI Uni Prot/ Swiss-Prot database (Figure 4.3.2.B.) showed greater diversity of identified homologues. Approximately 88 % of contigs aligned with Uni Prot/ Swiss-Prot homologues (77 % known and 11 % hypothetical proteins), leaving only 12.6 % unknowns (Figure 4.3.2.B.). An abundance of transcripts are involved in physiological and structural processes for e.g. photosynthesis, Calvin cycle transcripts, nucleic acid and protein synthesis. On the other hand, homologues related to stress and defense proteins appeared to be relatively low.

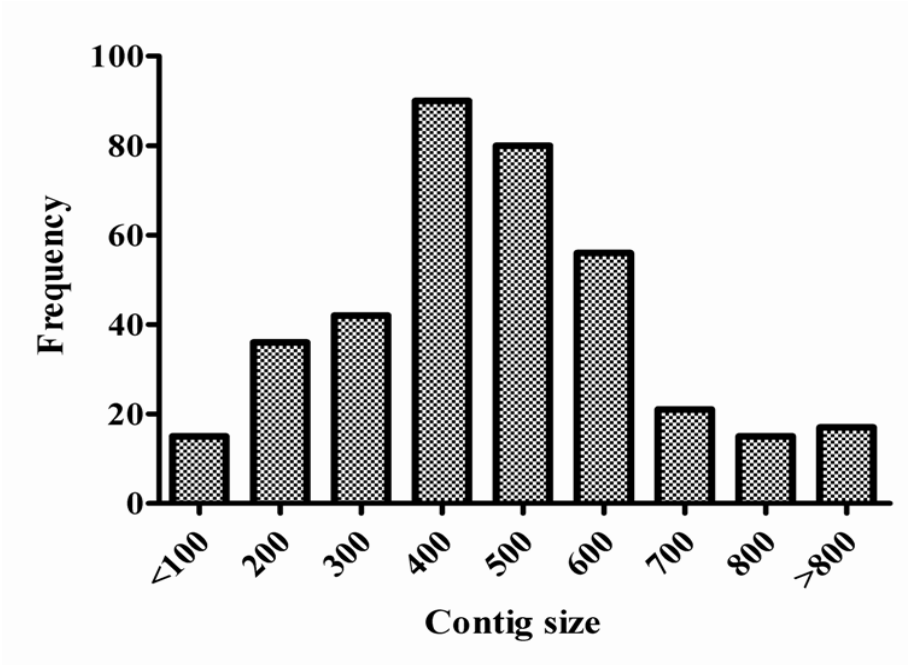

Figure 4.3.1. Overview of *Gelidium pristoides* contigs. Graph depicts size distribution range of obtained Contigs as well as frequency of occurrence in a certain size range.

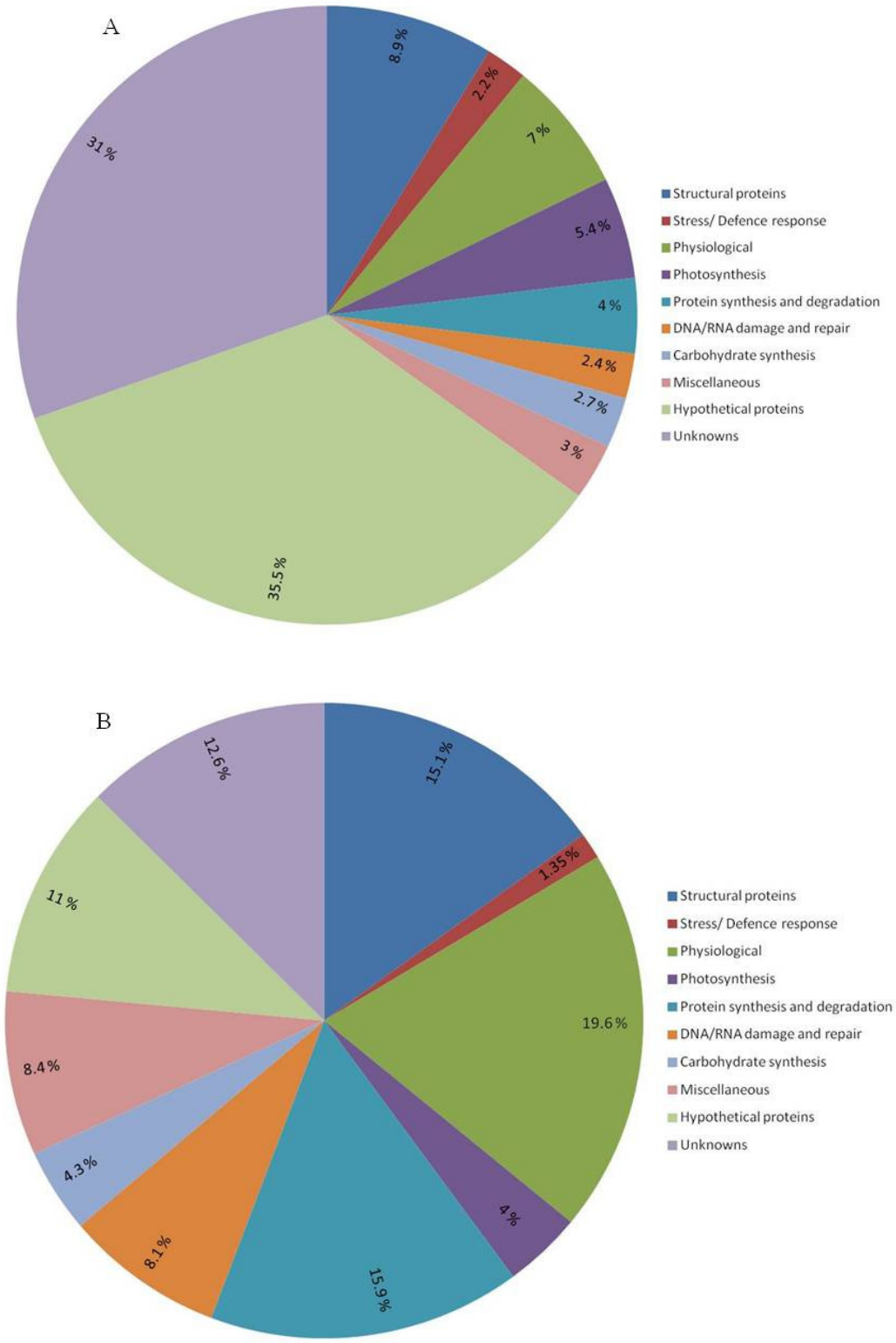

Figure 4.3.2. Multiple eukaryotic BLASTX analyses of assembled *Gelidium pristoides* contigs. Eukaryotic homologues were identified using the (A) Non-redundant BLAST and (B) Uni Prot/ Swiss-Prot queries. Homologous transcripts were sub-divided into metabolic categories according to cellular function. Percentage calculations for each category were performed using the number of transcripts divided by the total number of contigs.

4.3.3. *Gelidium pristoides* transcripts show homology to starch biosynthesis, starch catabolism and glycolysis related enzymes

While a plethora of transcripts are involved in general cellular activity, contigs showing homology to carbohydrate active enzymes are of particular interest to this study. BLASTX searches identified transcripts linked to starch or glygogen biosynthesis such as a 1,4-alpha-glucan branching enzyme (Contig00338) and glycogen synthases (Contig00266 and Contig00288) (Table 4.3.2.). In algae, these candidate genes are likely involved in floridean starch biosynthesis (Weber *et al*., 2004). Homology searches also revealed putative starch hydrolysis enzymes such as glucosidases. For example, Contig00010 showed homology to an *A. thaliana* peroxisomal beta-glucosidase 26 (EC 3.2.1.21), an enzyme involved in cleaving of terminal, non-reducing beta-D-glucose residues for the release of free beta-D-glucose. Interestingly, Contig00103 showed identity to a putative phosphoglucan phosphatase gene (LSF1) (EC 3.1.3.-). The LSF1 protein has been shown to play a substantial role in starch degradation from *A. thaliana* (Comparot-Moss *et al*., 2010).

Seven transcripts homologous to glycolytic pathway enzymes (Table 4.3.2.) were detected (See Figure 4.3.3.). Sequences for hexokinase and glucose transporting enzymes were however absent. The absence of hexokinase transcripts was not surprising as the enzyme was also not detected amongst *G. sulphuraria* ESTs (Weber *et al*., 2004). Amongst our transcripts, we identified possible phosphoglucomutase (EC 5.4.2.2) and phosphoglucoisomerase (EC 5.3.1.9) enzymes linked to glycolytic and cellular respiration activity. Collectively, transcripts showed highest homology to bacterial sequences with the exception of Contig00141, which shared significant homology (93 %) with red algae *Chondrus crispus* glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.59) (Table 4.3.2.).

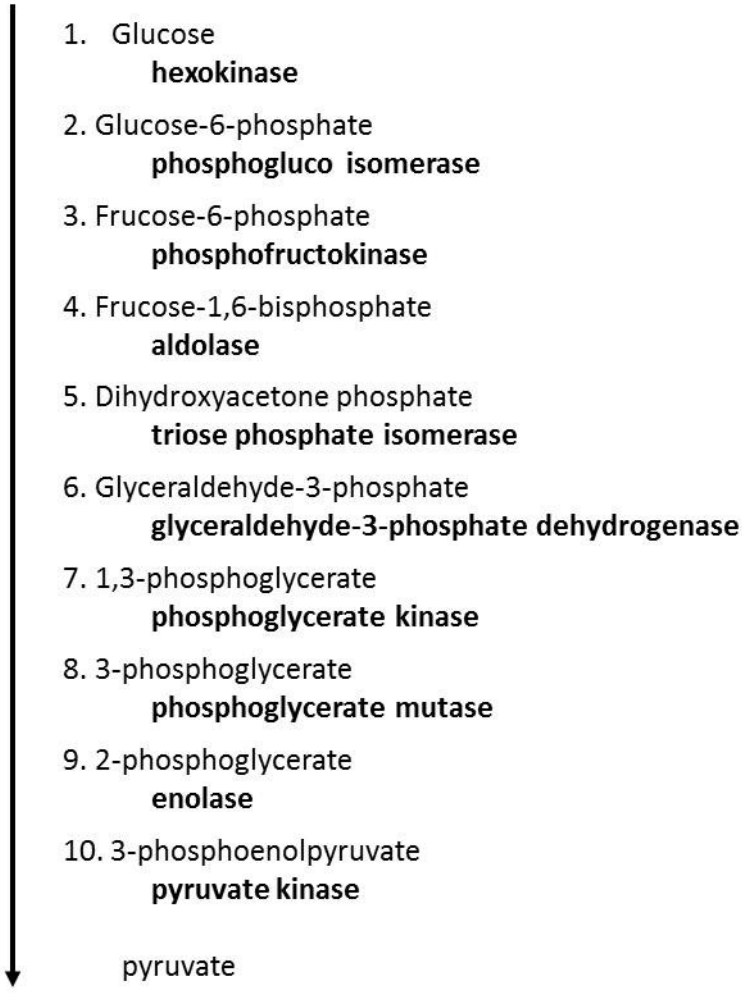

Figure 4.3.3. The enzymes of the glycolytic pathway.

4.3.4. The presence of putative cell wall modification enzymes of *G. pristoides*

No homologues related to galactan synthesis, for example β-1,3- and α-1,4-galactosyltransferases, were detected amongst *G. pristoides* contigs. Several transcripts related to hydrolases and cell wall modifying enzymes were however detected. For example, contigs 00235, 00131, 00135, 00193 showed similarity to plant and bacterial glycosyl hydrolases (Table 4.3.2.). Specific alignments of these sequences to known NCBI homologues illustrated contigs 00235 and 00135 resembled *Medicago truncatula* cell wall-associated hydrolases, 32 % and 63 % homology, respectively. On the other hand, contig00193 displayed a 30 % homology to an uncharacterized *Pseudoalteromonas citrea* cell-wall hydrolase. Although all three contigs showed cell wall-associated hydrolase BLAST hits, contig00193 did not align well with contigs 00235 and 00135, sharing only an overall homology of only 26 %. A putative cellulose synthase-like (Contig00091) was also identified

and displayed 50 % homology to red algae cellulose synthase A (EC 2.4.1.12) from *Griffithsia monilis*. Other cell wall-associated BLAST hits included extracellular matrix proteins such as a von Willebrand factor (vWA) D (Contig00220).

Table 4.3.2. Annotation of transcripts related to carbohydrate metabolism in *G. pristoides*.

Identified homologues	contigs	Contig size	E-value	Closest homology	Accession number
Glycolysis					
Glyceraldehyde-3-phosphate dehydrogenase	Contig00141	488 bp	3-47	*Chondrus crispus*	CAA51514
Fructose-1,6-bisphosphate aldolase	Contig108	463 bp	4e-34	*Ewardsiella ictaluri*	O52402
Phosphoglycerate kinase	Contig00022	200 bp	1.46e-25	*Aquifex aeolicus*	O66519
Glucose-6-phosphate isomerise	Contig00165	386 bp	4e-05	*Prochlorococcus marinus*	Q7V1I1
Pyruvate kinase	Contig00265	629 bp	7e-05	*Clostridium perfringens*	Q46289
Phosphoglucomutase	Contig00042	533 bp	3.3e-23	*Arabidopsis thaliana*	Q9SCY0
Uncharacterized phosphosugar isomerase	Contig00143	825 bp	1.90e-10	*Bacillus subtilis*	O67500
Starch biosynthesis					
1,4-α-glucan branching enzyme GlgB	Contig00103	510 bp	3e-05	*Lactobacillus acidophilus*	Q5FL68
1,4-α-glucan branching enzyme GlgB	Contig00338	121 bp	1e-04	*Magnetospirrilum magneticum*	Q2W2Q6
Glycogen branching protein	Contig00288	292 bp	1e-06	*Hirschia baltica*	YP_003060259
Glycogen synthase	Contig00266	215 bp	3e-05	*Alkalilimnicola ehrlichei*	Q0AA27

Table 4.3.2. continued.

Starch catabolism					
Alpha-amylase	Contig00358	196 bp	4e-04	*Hordeum vulgare*	P04750
Sucrase/ Isomaltase	Contig00087	497 bp	0.006	*Rattus norvegicus*	P23739
Beta-glucosidase, peroxisomal	Contig00010	536 bp	2e-04	*Arabidopsis thaliana*	O64883
Probable beta-glucanase	Contig00132	546 bp	6e-06	*Aspergillus fumigatus*	Q8J0P4
Phosphoglucan phosphatase	Contig00103	510 bp	9e-17	*Arabidopsis thaliana*	F4J117
Cell wall modification					
Cell wall-associated hydrolase	Contig00235	2131 bp	2e-39	*Medicago truncatula*	XP_003637074
Cell wall-associated hydrolase	Contig00135	2284 bp	1e-26	*Microscilla marina*	ZP_01689674
UDP-arabinopyranose mutase 1	Contig00027	389 bp	0.003	*Arabidopsis thaliana*	Q9SRT9
Alpha-1,3-galactosidase	Contig00149	482 bp	1e-04	*Bacteriodes fragilis*	Q5LGZ8
Cellulose synthase-like protein	Contig00091	528 bp	0.004	*Oryza sativa*	Q9LHZ7
Xyloglucan endotransglycosylase	Contig00131	553 bp	3e-06	*Musa acuminata*	ACQ85269
Putative membrane-bound metal-dependent hydrolase	Contig00193	887 bp	7.39e-3	*Pseudoalteromonas citrea*	ZP_10273920
von Willebrand factor D and EGF domain-containing protein	Contig00220	193 bp	1.90e-03	*Homo sapiens*	Q8N2E2

4.4. DISCUSSION

The G. pristoides transcriptome was sequenced to identify sulfated galactan biosynthesis enzymes. Sulfated polysaccharide biosynthesis enzymes (e.g. sulfotransferases, sulfurylases, -β-1,3- and α-1,4-galactosyltransferases) have not to date been identified from red algae. A *de*

novo sequencing approach was followed due to greater probability of identifying these elusive transcripts. Transcripts encoding general carbohydrate metabolism genes i.e. glycolysis, starch biosynthesis, starch catabolism and Leloir pathway associated homologues were also of interest. BLASTX analyses of *G. pristoides* contigs were queried against NCBI non-redundant database (Altshul *et al*., 1990). Using this approach, no sulfated galactan biosynthesis enzymes were detected. Identical outcomes were obtained by Collén and colleagues investigating ESTs from regenerated *Chondrus crispus* (Rhodophyta) protoplasts (Collén *et al.,* 2006). We ascribed this lack of sulfated galactan-associated genes to poorly annotated red algae sequences on NCBI. Red algae DNA sequences are routinely annotated using land plant reference sequences. However, red algae share little homology with terrestrial plants. This is evident from NCBI where genomic sequence data is available for only two red algae species, *Guilardia theta* (0.5 Mb) and *Cyanidioschyzon merolae* (17 Mb), respectively (Douglas *et al*., 2001; Matsuzaki *et al*., 2004; Weber *et al*., 2006). Similar conclusions have been made in studies focusing on annotation of red algae sequence (*Gracilaria gracilis*, *Galdieria sulphuria* and *Griffithsia okiensis*) (Lluisma and Ragan, 1997; Weber *et al*., 2004; Lee *et al*., 2007). Transcripts for cellulose synthase (contig00091), extracellular matrix von Willebrand protein (contig00220) and cell wall-associated hydrolases (contig00131, contig00135, contig00193 and contig00235) indicate stimulation of cell well generation i.e. possible sulfated galactan production. Observations made by Collén and co-workers (2006), while investigating cell wall generation in *Chrondrus crispus* protoplasts, showed an abundance of similar transcripts (cellulose synthases, von Willebrand proteins and cell wall-associated hydrolases). They showed that these transcripts coincided with cell wall generation (in protoplasts) and concluding that these putative proteins have possible association with the extracellular matrix in *Chrondrus crispus*. Although, putative homologues to cell wall-associated proteins were obtained, it cannot be certain that these enzymes are involved in sulfated galactan biosynthesis.

Despite the absence of sulfated galactan-associated transcripts, our results highlight events preceding agar production through detection of starch biosynthesis and starch catabolism homologues (Table 4.3.2). As the main storage polysaccharide in red algae, floridean starch production increases with increasing iridescence (Hemmingson *et al*., 1996). In contrast to terrestrial starch (amylase and amylopectin), floridean starch consists exclusively of amylopectin derived from UDP-D-glucose and UDP-D-glucose pyrophosphorylase (UGPase) (Viola *et al*., 2001; Deschamps *et al*., 2008; Dauvillée *et al*., 2009). Although *G. pristoides* UGPases were not detected, other starch biosynthesis associated enzymes were present i.e.

phosphoglucomutase, glycogen synthase and α-1,4-glucan branching enzyme transcripts, suggesting floridean starch production at the time of sampling (dawn). Interestingly, UGPase transcripts were also not identified by Barbier and co-workers (2005) amongst ESTs of micro red algae *G. sulphuraria* ESTs and *Cyanidioschyzon merolae* .

In red algae, nocturnal floridean starch hydrolysis coincides with galactan production (Fournet *et al*., 1999). As sampling was confined to morning hours in the presence of low light, transcripts related to starch hydrolysis (α-amylase, β-glucanase, β-glucosidase and phosphoglucan phosphatase) were still present. In terrestrial plants, it is well known that starch hydrolysis produces glucose during sequential degradation steps (Smith *et al*., 2005). Even though starch degradation in red algae has yet to be fully elucidated, it is expected that floridean starch degradation is analogous to terrestrial plants (Ball *et al*., 2003; Ball *et al*., 2011). In light of our transcriptomic data, it is presumed that α-amylase, β-glucanase, β-glucosidase and phosphoglucan phosphatase enzymes participate in floridean starch degradation. Our hypothesis is further strengthened by the previous work of Barbier and colleagues (2005) that identified similar ESTs related to starch hydrolysis enzymes (β-glucosidase, α-amylase and various phosphatases) in *G. sulphuraria* and *Cyanidioschyzon merolae* (Barbier *et al*., 2005). Detection of starch degradation transcripts amongst *G. pristoides* cDNAs suggests floridean starch hydrolysis metabolism of glucose. The identification of phosphoglucan phosphatase and α-amylase transcripts suggests initiation of starch degradation and liberation of glucose in *G. pristoides*, respectively. It is expected that the liberated glucose, despite entering glycolysis, would also be activated to UDP-D-glucose. The Leloir enzyme, UDP-galactose 4-epimerase subsequently converts the nucleotide sugar to UDP-D-galactose.

UDP-D-galactose is the major source of galactose required for sulfated galactan production (Goulard *et al*., 1999; Goulard *et al*., 2003). Our earlier investigations (Chapter 3) showed high nocturnal activity of UGE. We hypothesized that elevated UGE expression corresponds to nocturnal floridean starch hydrolysis, ultimately contributing to galactan production (Chapter 3). UGE appears to play an intimate role in supplying UDP-D-galactose for galactan production. UDP-D-galactose is also the most abundant nucleotide sugar in red algae, indirectly emphasizing the role of UGE as primary supplier of UDP-D-galactose (Manley and Burns, 1991; Fournet *et al*., 1999; Goulard *et al*., 1999; Goulard *et al*., 2003). UGE involvement in production of sulfated galactan was also demonstrated for ascidian species

Styela plicata (Mourão, 1990). Despite *de novo* sequencing yielding no UGE transcripts, its Leloir counterpart UDP-D-galactose-1-phosphate uridylyltransferase (Galt) was detected.

Correspondingly, UGE was also not detected amongst *C. crispus*, *Griffithsia okiensis* and *G. gracilus* ESTs, although Galt transcripts were described for *G. gracilus* and *G. sulphuraria* (Lluisma and Ragan, 1997; Collén *et al*., 2006; Lee *et al*., 2007). However, an abundance of UGE transcripts were identified from *G. pristoides* cDNA libraries (Chapter 2). In addition, SqPCR amplification of partial UGE fragments showed presence of the enzyme in the *G. pristoides* RNA population (See Chapter 2 and Chapter 3). We attribute the lack of UGE transcripts to a combination of harvesting time (during ambient daylight hours) and limited quantities of transcriptome sequence data.

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CHAPTER 5

General discussion

5.1. INTRODUCTION

Industrial applications of natural polysaccharides are currently gaining momentum due to increased global demand for natural products (Delattre *et al*., 2011). On the forefront of this revolution are sulfated polysaccharides produced by algae. Their unique physico-chemical properties lend rheology, thickness and texture to various foodstuffs and cosmetics (Radmer, 1996; De Ruiter and Rudolph, 1997; Tseng, 2001, Arad and Ontman, 2010, Delattre *et al*., 2011). Over the past 20 years sulfated polysaccharides have also attracted significant research attention as bioactive compounds for application as pharmaceuticals i.e. anti-coagulant and anti-thrombotic treatments as alternative to heparin sulfate (Mourão and Pereira, 1999; Mazumder *et al*., 2002; Smit, 2004; Delattre *et al*., 2011). In addition, these polysaccharides are furthermore potent anti-viral agents (Smit, 2004). In the Far East, algae (seaweed) have been an important source of nutrition for centuries and their health beneficial properties are well known (Tseng, 2001). In contrast, western countries have only recently discovered these benefits, especially for the production of sulfated polysaccharides (Delattre *et al*., 2011). Red algae in particular, are exploited for their sulfated galactans i.e. agarans and carrageenans. Agar and agarose is produced for application as molecular biology reagents with cumulative annual sales of $210 million in the 90's (Radmer, 1996). Red seaweed is mostly harvested from natural sources using traditional methods often resulting in industrial demand exceeding supplying capabilities. In the near future, over harvesting of natural populations may lead to depletion and extinction of industrially relevant species. In China, traditional methods of cultivation and harvesting of seaweed were replaced by biotechnology platforms in the 1950s leading to establishment of 6 major aquaculture industries in order to preserve natural populations (Tseng, 2001).

5.2. GENERAL DISCUSSION AND CONCLUDING REMARKS

Gelidium pristoides, a red algae species endemic to Kalk Bay, Cape Town was collected for this study. *Gelidium pristoides* is of commercial importance and harvested for its high quality

agar. In the 1980's, ecological concern was raised due to over-harvesting of natural seaweed populations (Anderson *et al*., 1990). To conserve South African red seaweed populations and prevent over-harvesting, we aimed to elucidate genes responsible for sulfated galactan (agar) biosynthesis in red algae for expression in a heterologous host. Expression cDNA libraries constructed from *G. pristoides* mRNA were functionally screened for genes encoding involved in galactan biosynthesis. Through functional complementation of an *E. coli GalE* (UDP-galactose 4-epimerase) mutant, our efforts resulted in the identification of a UDP-galactose 4-epimerase (UGE) encoding gene from *Gelidium pristoides* (*Gp*UGE). It is known from the Leloir pathway that the UGE enzyme is necessary for converting UDP-D-glucose to UDP-D-galactose for galactan production (Frey, 1996). Given the lack of genomic and amino acid sequence data for UGE enzymes from red seaweed, we consider this finding significant.

*Gp*UGE sequence showed similarity to known UGE sequences and possessed conserved domains consistent with UGEs characterized from *E. coli*, *S. cerevisiae*, *H. sapiens*, *A. thaliana*, *P. tricornutum* and *E. siliculosus*. The enzyme has an apparent K_m of 0.27 mM and an observed V_{max} of 0.015 µmol/min/mg protein. No kinetic deviation from previously characterized UGEs was observed, apart from enhanced activity upon addition of exogenous NAD^+. *Gp*UGE functions optimally at 25 ^oC to 30 ^oC and between pH 8.5 and pH 9.0. RT-PCR analysis revealed elevated UGE expression levels during the night cycle. In conjunction with *de novo* transcriptome sequence analysis, RT-PCR analysis correlated UGE expression with floridean starch catabolism and agar synthesis in *G. pristoides*. Previous reports based on physiological data speculated UGEs are precursor enzymes for the production of sulfated galactans in red algae (Fournet *et al*., 1999; Goulard *et al*., 1999; Goulard *et al*., 2003). In prokaryotes and eukaryotes evidence suggests the involvement of this enzyme in cell wall polysaccharide synthesis (Joersbo *et al*., 1999; Seifert *et al*., 2002; Seifert, 2004; Barber *et al*., 2005; Zhang *et al*., 2006; Rösti *et al*., 2007; Singh *et al*., 2007).

Due to its established role in galactose metabolism, production of UDP-D-galactose and its involvement in cell wall polysaccharide synthesis, UGE presents an ideal target for bioengineering. Introduction of minor modifications by directed evolution or error-prone PCR approaches may increase kinetic turnover rates from UDP-D-glucose to UDP-D-galactose.

In conclusion, we identified a UDP-galactose 4-epimerase gene from red seaweed *Gelidium pristoides* and the enzyme was characterized. Our qPCR and Next generation sequencing results highlight a relationship between UGE expression, floridean starch degradation and galactan production in red algae. As far as our knowledge, this the first report of sequencing of and biochemical characterization of an UGE from red seaweed.

5.2. REFERENCES

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APPENDIXES

Appendix I

Section 1: Primers, plasmids and constructs

Table 2.2.3. PCR primers

Gene or plasmid name	Primer sequence	Origin
S. cerevisiae Gal10	Epimerase Fwd 5'-CCGGCACATCTGCGTTTCAGGAACGC-3' Epimerase Rev 5'-GGATGAGCCTTCGCTCAACAGTGC-3'	This study
A. thaliana GME**	GDP-Epi Fwd 5'-GGCCGAATTCATGGGAACTACCAATGAACA-3 GDP-Epi Rev2 5'-GGCCAGATCTTCACTCTTTTCCATCAGCCGC-3'	This study
A. thaliana UGE*	AtUGE Fwd 5'-CCGGCTGCAGATGGGTTCTTCTGTGG-3' AtUGE Rev 5'-CCGGGAATTCTCAAAGCTTATTCTGGTAACC-3'	This study
G. pristoides UGE*	GpUGE Fwd 5'-CCGGGGATCCATGACAACAAACAAGACAATTCTCG-3' GpUGE Rev 5'-GGCCCTCGAGTTATTTAGGACCATAACCATTTGG-3'	This study
G. pristoides Sq UGE	GpUGE_Sq Fwd 5'-CCGCTCCAATTTCAACATTCTCAGTTACTG-3' GpUGE_Sq Rev 5'-GGATGACAACAAACAAGACAATTCTCG-3'	This study
G. pristoides RuBisCo	GpRuBisCo Fwd 5'-GGTTCTATTGCTGCGACAGGC-3' GpRuBisCo Rev 5'-CCGGATACCACCAGATGCTACTGG-3'	This study
G. pristoides 18S	Gp18S Fwd 5'-GGCGTTTGCCT TGTACACATTAGC-3' Gp18S Rev 5'-GGCTGTCAATCCTCACTATGTCCG-3'	This study

* UDP-galactose 4-epimerase

** GDP-mannose 3',5'-epimerase

Table 2.2. Plasmids and constructs

Name	Selection	Origin
pBluescriptSK-	Amp/ X-Gal	Stratagene, USA
pSK-Clone 17	Amp/ X-Gal	This study I.P.B*
pRSET-A	Amp/ X-Gal	This study I.P.B*
pRSET-*At*UGE	Amp/ X-Gal	This study I.P.B*
pRSET-*Gp*UGE	Amp	This study I.P.B*
pGEM-T Easy	Amp/ X-Gal	Promega, USA
pGEM-*At*GME	Amp/ X-Gal	This study I.P.B*
pCEL13	Amp	IWBT**
pCEL13/*At*GME	Amp	This study I.P.B*
pJET	Amp	Ferments, USA
pJET-*Gp*UGE	Amp	This study I.P.B*
pJET-*At*UGE	Amp	This study I.P.B*
pJET-GpRuBisCo	Amp	This study I.P.B*
pJET-Gp18S	Amp	This study I.P.B*
pPVD1	HIS/Amp	IWBT**
YEplac112	TRP/ Amp/ X-Gal	Department of Microbiology, Stellenbosch University
pYES2.0	URA/ Amp	Invitrogen, Germany
pYES-*At*UGE	URA/ Amp	This study I.P.B*

* I.P.B. – Institute for Plant Biotechnology

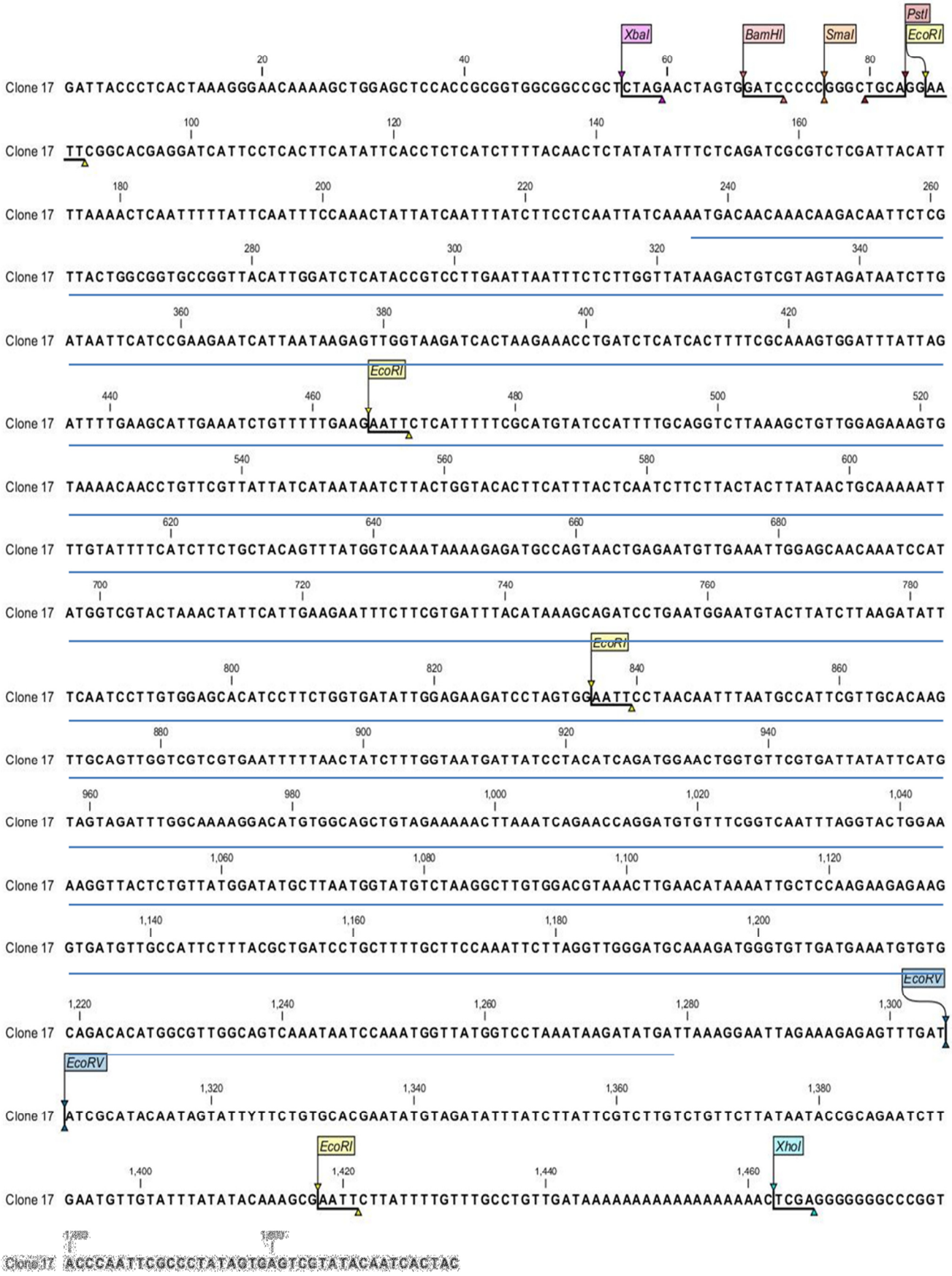
**I.W.B.T. – Institute for Wine Biotechnology

Appendix II

Section 1: DNA sequence annotations

1.1. *Gelidium pristoides* UDP-galactose 4-epimerase DNA (*Gp*UGE)

1.2. cDNA library Clone 17



Section 2: Amino acid sequences

2.1. *Gelidium pristoides* UDP-galactose 4-epimerase protein (*Gp*UGE)