SCREENING AND EVALUATING ENZYMES PRODUCED BY ACTINOBACTERIA GROWING ON SEAWEED AND THEIR BIOPRODUCTS

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DECLARATION

'I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.'

Thi Nhu Thuong Nguyen

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Tuesday, 14th April 2020

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ABBREVIATIONS

ul, ml, L	microlitre, millilitre, litre
uM, mM, M	micromolar, millimolar, molar
ug, mg, g	microgram, milligram, gram
%	percent
ANOVA	Analysis of Variance
ACN	Acetonitrile
ABC	Ammonium bicarbonate
BME	Beta Mercaptoethanol
bp	base pair
^{O}C	Degree Celsius
CPC	Cetylpyridinium chloride
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ESI-MS	Electron spray ionization–mass spectrometry
FPLC	Fast protein liquid chromatography
HPLC	High performance liquid chromatography
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDa	KiloDalton
LB	Luria-Bertani

LVA	Low viscosity sodium alginate
MQ water	Milli Q water
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
OD_{600}	Optical Density @ 600 nanometers
PCR	Polymerase Chain Reaction
PL	Polysaccharide lyase
PL7	Polysaccharide lyase family 7
RO water	Reverse osmosis
RMS	Response Surface Methodology
sp.	species (singular)
spp.	species (plural)
SDS-PAGE	Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis
SB	Sodium borate
TLC	Thin layer chromatography
TBE	Tris/Borat/EDTA
UV	Ultraviolet

ABSTRACT

Polysaccharide-degrading enzymes have played an important role in the discovery of biological functional unsaturated oligosaccharides. Although a large number of such enzymes have been reported, the enzymes extracted from actinobacteria that can degrade polysaccharides from seaweed are rather limited. This study was carried out to screen, purify and characterise native enzymes from actinobacteria grown in seaweed media to determine whether actinobacteria can be a good source for producing potentially novel polysaccharide-degrading enzymes. Moreover, the recombinant enzymes were prepared to meet the requirements of commercial polysaccharide-degrading enzymes for industrial processes.

Eighty strains of actinobacteria isolated from decomposing seaweed have been screened for polysaccharide-degrading enzymes. The strains DS40, DS44 and DS79, which were identified as *Streptomyces griseorubens, Streptomyces luridiscabiei,* and *Streptomyces sundarbansensis* respectively, could produce the highest activity of alginate lyase when they were cultured in a seaweed containing medium. The DS40, DS44, and DS79 alginate lyase were purified by combining anion exchange chromatography and size exclusion chromatography with the specific activity of 67.75 U/mg, 108.6 U/mg and 103.4 U/mg, respectively. These enzymes have the molecular weight of approximately 29 kDa. The alginate lyase genes of strains DS40, DS44 and DS79 were composed of 780 bp encoding 259 amino acid residues. There was a 100% similarity in the amino acid sequences of DS40 and DS79 alginate lyase leading to the same type of alginate lyase. DS44 and DS79 alginate lyase exhibited the activity towards both polyguluronate and

polymannuronate, indicated that they are bifunctional alginate lyases, however, they preferentially degraded polyguluronate more so than polymannuronate. The optimal pH of both enzymes was 8.5 and optimal temperatures were 45^{o}C and 55^{o}C for the DS44 and DS79 alginate lyase, respectively. They also were grouped into salt-tolerant alginate lyases and showed optimal salt concentration at 0.6 M. Metal ions Mn^{2+}, Co^{2+}, and Fe^{2+} which increased the alginate degrading activity and by contrast, the enzymes were inhibited in the presence of Zn^{2+} and Cu^{2+}. The highly conserved regions of their amino acid sequences suggested that DS44 and DS79 were alginate lyases of polysaccharide lyase family 7. The ESI- MS analysis showed that the main oligosaccharides break-down products were disaccharides, trisaccharides and tetrasaccharide, which indicated that these enzymes acted as endo type alginate lyases. The amino acid sequence of DS44 showed more than 10% difference to a reported alginate lyase, which demonstrated that DS44 alginate lyase from *Streptomyces luridiscabiei* could be novel and a potential enzyme for efficient productions of alginate oligosacharides with low degrees of polymerization.

The genes encoding DS44 and DS79 alginate lyases were cloned using the plasmid vector pColdI and expressed in *Escherichia coli* BL21 (DE3). The optimal IPTG concentrations for induction at 15^{o}C in the *E. coli* expression system were 0.1 M and 1 M for DS44 and DS79 alginate lyase, respectively. The His-tagged proteins were purified effectively by using a Ni^{2+} Sepharose affinity chromatography and refolded efficiently with a linear gradient of urea (8-0 M). The recombinant DS44 and DS79 alginate lyase exhibited the specific activities of 80.82 U/mg and 78.54 U/mg, respectively. The optimal pH and temperature of the recombinant DS44 and DS79 enzymes were pH 8.5, 45^{o}C; and pH 7.5, 55^{o}C, respectively. Therefore, cloning and

expression of recombinant protein is a powerful technique for producing commercial alginate lyase as a potential candidate for use in various applications in the food, chemical, medical, and biological industries.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Seaweed

1.1.1 Overview of seaweeds

Seaweeds are known as macroalgae or multicellular marine algae which have forms that resemble terrestrial plants. They are represented in three groups as red algae (Rhodophyta), brown algae (Phaeophyceae) and green algae (Chlorophyta), based on their colours (Wijesinghe & Jeon, 2012). The red seaweed colour is because of the phycoerythrin pigment, and includes *Porphyra, Gracilaria, Gelidiella* and *Euchema*. The green seaweed colour results from the dominance of chlorophyll in genera including *Enteromorpha, Caulerpa* and *Ulva.* The pigmentation from fucoxanthin effects a brown colour in seaweeds including *Laminaria* and *Undaria* (Lee, 2008). The brown and red algae are unique in that they are considered to be marine, whereas green algae are collected in freshwater as well as marine environments. Examples of red, green and brown seaweeds are shown in Figure 1.1. They generally grow on rocks or other hard substrata in coastal areas (Lewis, 1964). There are approximately 25,000–30,000 species (Santos *et al.*, 2015), with a variety of sizes and forms of seaweeds in which brown algae is the largest and most complex seaweed group including bull kelp, wracks and sargassum (Hoek *et al.*, 1995).

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Figure 1.1 Red, green and brown seaweed

1.1.2 Constituents of seaweeds

Table 1.1: Relative proportions of the main constituents of marine seaweed (Kim *et al.,* 2011).

Contents	Green algae	Red algae	Brown algae
Example species	*Enteromorpha* spp.*,* *Codium fragile*	*Porphyra tenera, Gelidium amansii*	*Undaria pinnatifida, Laminaria* spp.*, Hizkia fusiforme, Sargassum fulvellum*
Dry matter (%) Carbohydrates (%) Protein (%) Lipids (%)	20 48-55 51-15 1-5	30-40 53-70 7-15 1-5	5-10 45-60 38-51 1-5

Compared with terrestrial plants, seaweeds have a higher protein content of approximately 38 – 51% and alternating levels of carbohydrates (Table 1.1). Carbohydrates are the major constituents of seaweeds, accounting for 45–70%. Seaweed carbohydrates consist mostly of alginate, fucoidan and laminarin (Sakatoku et al., 2012) which are all bioactive compounds which are considered to be attractive for commercialisation and structural research.

Seaweeds have been recognised to produce chemically and functionally novel secondary metabolites including antioxidants, anticoagulant, antivirus, antimicrobial, anti-tumor and anticancer agents (Manilal *et al.*, 2009). Therefore,

they can be considered as potential natural sources of bioactive compounds that have a wide range of industrial applications such as functional food and nutraceutical products.

1.1.3 The Global and Australian seaweed industry

Total global production of seaweeds was approximately 15.8 million tons produced by aquaculture and 1.1 million tons collected from the ocean in 2018 (FAO 2010). Seaweed products which are used as a food source are considered the largest market with a value which is estimated to be US$6 billion (Sakatoku *et al.*, 2012). The largest supplier of seaweeds in Asia is China, estimated to produce over 90% of the world total yield (Lee, 2010). Annually, the Japanese industry produces in excess of 200,000 tons of Wakame (*Undaria pinnatifida*) (Sakatoku *et al.*, 2012). In Korea, the seaweed production level was seen to be approximately 882,000 tons for two years from 2008 to 2010, mostly by aquaculture (Kim *et al.*, 2013). The major species produced in these countries were brown seaweeds, including *Laminaria japonica* and *Undaria pinnatifida.*

In comparison, the seaweed industry in Australia is significantly less developed in terms of commercial production. McHugh and King (1998) reported that the seaweed industry in Australia is relatively localised and small, and few seaweed species are commercially exploited. However, Australia currently imports almost $20 million of seaweed per year (MISA, 2011), while Southern Australia is estimated to produce approximately $7 million of the local seaweed production (Budarin *et al.*, 2011).

Southern Australia has been identified as a seaweed biodiversity hotspot (Lorbeer *et al.*, 2017; Phillips, 2001), characterised by long stretches of coastline, clean water and a favourable climate that leads to an abundance growth of seaweed (Lorbeer *et al.*, 2013). It is reported that 62% of the 1200 species of seaweed is native to Southern Australia and do not occur anywhere else in the world (APCAP, 2012). However, the seaweed industry in South Australia is currently limited, with the majority of harvested seaweed being utilised as low value agricultural products, including fertilizers from beach-cast wrack and imported biomass (Lorbeer *et al.*, 2013). Seaweeds contain high value polysaccharides, particularly alginates and fucoidans with the potential for applications in functional foods, cosmetics and pharmaceutical development. However, limited research and development has been done with this unique natural resource.

1.2 Bioactive polysaccharides from seaweed

1.2.1 Alginate

1.2.1.1 Structure of Alginate

Alginate is the most abundant polysaccharide located in the matrix and in the cell wall of brown seaweed. It is composed of α-L-guluronate (G) and β-D-mannuronate (M) as the major monomeric units (Wong *et al.*, 2000) (Figure 1.2a). These units are organized in three different blocks, poly α-L -guluronate (polyG), poly β-D-mannuronate (polyM) and the heteropolymer (polyMG) (Haug *et al.*, 1967) (Figure 1.2b). The number of G and M blocks as well as the ratio of G to M within alginates are varied and depend on the sources from which they are isolated. Lee and

Mooney (2012) summarised that more than 200 different alginates currently are being manufactured. The structure of alginate is variable, presenting two types of homopolymeric sequences (MM and GG) as well as heteropolymeric sequences (MG and GM) (Andriamanantoanina & Rinaudo, 2010).

Alginates generally have molecular weights ranging from 500 kDa to 1000 kDa (Rioux *et al.*, 2007). Alginates liberated from brown seaweeds, comprising up to 40 – 47% of dry biomass (Holdt & Kraan, 2011). For example the alginate contents are 22 to 30% of the dry weight in *Ascophyllum nodosum* and 25 to 44% for *Laminaria digitata* (Haug *et al.*, 1967).

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Figure 1.2: Chemical structure of alginate: (a) chain conformation and (b) block distribution. This figure has been reproduced from Pawar & Edgar (2012).

1.2.1.2 Sources of Alginate

All commercial alginates are derived from marine algae. They are extracted from biomass of the brown algae such as *Laminaria hyperborea, Laminaria digitate, Macrocystis pyrifera, Ascophyllum nodosum, Eclonia maxima Laminaria japonica, Lessonia nigrescens, Sargassum sp*., and *Durvillea antarctica* (Kim *et al.*, 2011). Furthermore, alginate can also be synthesized by some bacteria including *Pseudomonas aeruginosa* (Albrecht & Schiller, 2005*), Azotobacter vinelandii (Clementi, 1997), Azotobacter chroococcum* (Pecina & Paneque, 1994), *P. fluorescens* and *P. putida* (Conti *et al.*, 1994).

1.2.1.3 Applications

Recently, commercial alginates extracted from seaweeds are used in the food, pharmaceutical and biotechnological industries. When utilised as a gelling agent, due to its high content of guluronic acid, alginate can form a strong gel in the presence of calcium ions. It can also immobilize cells for many biotechnological applications. Moreover, the viscosity of cosmetics are increased due to the addition of alginate. It can be applied in functional food for diet control due to the indigestible nature of alginate in the human gastrointestinal tract (Kim *et al.*, 2011).

Additionally, alginate is also used in pharmaceutical formulations including indigestion tablets and chelators which help to remove radioactive toxins and heavy metals in the human body. Moreover, hydrogels for cell encapsulation made from alginate has been one of the most advanced biomedical applications (Lee & Mooney, 2012; Pawar & Edgar, 2012).

1.2.2 Fucoidan

1.2.2.1. Structure of fucoidan and its distribution

The first report of fucoidan from marine brown algae was made 104 years ago by Kylin (1913). This polysaccharide liberated from the cell wall of brown algae was predominantly composed of sulphated L-fucose. Later on, Vasseur (1948) demonstrated that fucans also occurred in marine invertebrates such as in the egg jelly coat of sea urchins or in the body wall of sea cucumber. To date, other natural sources of fucoidan have not been reported (Berteau & Mulloy, 2003).

Fucoidan belongs to a family of sulphated homo- and heteropolysaccharides. These polysaccharides are composed of a high content of glucuronic acid and low content of fucose or built as sulphated fucose. In some fucoidans, their structure also consists of galactose, xylose and mannose. Therefore, structures of fucoidan are extremely diverse because of the variety of brown seaweed species and marine invertebrates that produce them (Berteau & Mulloy, 2003; Kusaykin *et al.*, 2008) (Figure 1.3). For example, in brown seaweed, fucoidan from the orders *Chordariales* and *Laminariales* consists of residues of 1,3-α-L Fucopyranose in the main chains (Bilan *et al.*, 2002; Chizhov *et al.*, 1999), the side chains comprising fucose (*Chorda filum*) or D-glucuronate (*Cladosiphon okamuranus*) residues, attached to the main chain in the C2 position (Figure 1.3 C). Another structure of fucoidans from the order *Fucales* (*Cyclosporophyceae*) is constructed of alternating 1,3- and 1,4-linked α-L-fucosyl residues (Bilan *et al.*, 2004; Chevolot *et al.*, 2001; Descamps *et al.*, 2006).

Chevolot *et al.* (2001) noted that the most common structure of fucoidans from both *Ascophyllum nodosum, Fucus evanescens* and *Fucus vesiculosus* is the disaccharide repeating unit [4)- α-L-Fucp(2,3di-OSO_3^-)-(1,3-α-L-Fucp(2OSO_3^-)-(1] (Chevolot *et al.*, 1999; Chevolot *et al.*, 2001) (Figure 1.3 A). Nishino *et al.* (1991) showed the 3-linked, preponderantly 4-sulphated fucoidan from *Ecklonia kurome* (Figure 1.3 B).

Figure 1.3: Regular structures of fucoidans from several species of brown algae. This figure has been reproduced from Albrecht & Schiller (2005).

In contrast with algal fucoidans, the polysaccharides from marine invertebrates consist of a repeating unit, either mono-, tri-, or tetrasaccharide that are defined by

a distinct pattern of sulphate (Berteau & Mulloy, 2003). Generally, each species has its own pattern. For example, *Strongylocentrotus droebachiensis* and *Strongylocentrotus purpuratus* produce two distinct types of sulphated fucans. In contrast, *Arbacia lixula* and *Strongylocentrotus droebachiensis* share a common structure of fucan (Figure 1.4)

The molecular weight of fucoidan is approximately 100 kDa (Rioux *et al.*, 2007) with the amount of fucoidan in algae at about 25- 30% of its dry mass (Kusaykin *et al.*, 2008). The bioactivities of fucoidan depend on its molecular weight. The low molecular weight fucoidan has a higher degree of bioactivity in comparison with high molecular fucoidan (Song *et al.*, 2012).

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Figure 1.4: Repeating subunits of sulphated fucans and a sulphated L-galactan from sea urchin egg jelly. This figure has been reproduced from Berteau & Mulloy (2003).

1.2.2.2. Biological properties of fucoidans

Fucoidans extracted from seaweeds have recently been employed as nutraceuticals in the USA and Australia. This is due to their diverse biological activities such as immunomodulatory, antitumor, antibacteria, antiviral, anti-inflammatory, anticoagulant, and anti-thrombotic effects (Kusaykin *et al.*, 2008). For example, fucoidans from brown seaweeds *Laminaria cichorioides* and *Fucus evanescens* act as anticoagulants and can inhibit thrombin (factor IIa) and factor X. The activation of the blood fibrinolytic system has been demonstrated by fucoidan from *Fucus evanescens* both in *in vitro* and *in vivo* experiments (Kusaykin *et al.*, 2008).

1.3 Polysaccharide-degrading enzyme from seaweed

1.3.1 Alginate lyase

1.3.1.1 Source and Classification

Characterized as being either mannuronate or guluronate lyases, alginate lyase cleaves the glycosyl linkages of alginate by the β-elimination mechanism which results in the production of oligosaccharides with an unsaturated uronic acid (Rahman *et al.*, 2010). Alginate lyases have been extracted from multiple sources such as marine algae, marine mollusks (*Littorina* spp., *Turbo cornutus*, *Haliotis* spp.) (Zhu & Yin, 2015), and a wide range of marine microorganisms (bacteria and fungi) and marine invertebrates. The following bacteria have been reported as producing enzymes that can degrade brown algae polysaccharides: *Alginovibrio aquatilis, Azotobacter vinelandii, Pseudomonas aeruginosa, Pseudomonas maltophilia, Flavourbacterium* sp., *Vibrio* sp. (Kim *et al.*, 2013). Schaumann and Weide (1990) demonstrated that there are four species of marine fungi which have the ability to degrade alginate and produce alginate lyase such as the deuteromycetes: *A. cruciatus, D. arenaria, D. salina,* and the ascomycete *C. intermedia*. In recent years, alginate lyase isolated from other strains of marine microorganisms have been developed to discover novel polymers of alginate for various applications in agricultural, industrial and medical fields (Zhang & Kim, 2010).

Alginate lyases can be classified into two groups according to their substrate specificities - there are polyG-block specific lyase and polyM-block specific lyase (Zhu & Yin, 2015). The majority of alginate lyases are observed to have the activity of polyM-specific lyase (Kim *et al.*, 2011). However, some alginates lyases can

degrade both polyM and polyG-block (polyMG-specific lyase) that show more bifunctional activity. Otherwise, alginate lyase which can also be classified as endolytic and exolytic alginate lyase, based on their mode of action (Wong *et al.*, 2000). Glycosidic bonds broken down by endolytic alginate lyases inside alginate polymers, result in the liberation of the main products of unsaturated oligosaccharides (di-, tri-, and tetra-saccharides). Alternatively, exolytic alginate lyase can additionally degrade oligosaccharides resulting in monomers (Kim *et al.*, 2012; Ochiai *et al.*, 2010; Park *et al.*, 2012). In recent years, there have been few reports and characterisations of exo-type lyases. Moreover, alginate lyases can also be classified into three types based on their molecular weight: small size (25–30 kDa), medium size (around 40 kDa), and large size (>60 kDa) (Zhu & Yin, 2015).

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Figure 1.5: The substrate specificity of alginate lyase. M: β-D-mannuronic acid, G: α-L-guluronic acid. This figure has been reproduced from Zhu *et al.* (2018).

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Figure 1.6: The degradation of alginate by using endolytic (A) and exolytic (B) alginate lyases. This figure has been reproduced from Ochiai *et al.* (2010)**.**

1.3.1.2 Substrate specificity and characterisation

Substrate specificity of alginate lyases is variable depending on the source of the lyase-producing organism (Table 1.2). For example, alginate lyase isolated from Eisenia *bicyclis, Pelvetia canaliculata, Dolabella auricular, Alteromonas* sp. strain H-4 preferred a polyM substrate while lyase from marine organism ATCC433367 exhibited activity only for polyG. However, *Spatoglossum pacificum* and

Pseudomonads (marine no. 8 and 9) produced lyases that display multiple substrate specificity including polyM, polyG and poly MG. Therefore, it has been suggested that these organism may produce more than one lyase or the enzymes may exhibit activity with multiple substrates. The characterisation of alginate lyases from different marine microorganisms is summarised in Table 1.3.

Table 1.2: Localisation and substrate specificity of alginate lyase from algae and marine organisms.

Source	Localisation	Substrate specificity	Action mode	Reference
Algae				
Eisenia bicyclis	Fronds	PolyM	Endolytic	(Shiraiwa *et al.*, 1975)
Pelvetia canaliculata	Intracellular	PolyM	Endolytic	(Madgwick *et al.*, 1978)
Spatoglossum pacificum	Fronds	PolyG & PolyM	Endolytic	(Shiraiwa *et al.*, 1975)
Invertebrates				
Dolabella auricula	Hepatopancreas	PolyM	Endolytic	(Nisizawa *et al.*, 1968)
Haliotis corrugata	Hepatopancreas	Alginate I: M	Endolytic	(Islan *et al.*, 2013)
Haliotis rufescens	Hepatopancreas	Alginate II: G	Exolytic	(Islan *et al.*, 2013)
Marine bacteria				
Alteromonas sp. Strain H-4	Extracellular	PolyM	Endolytic	(Sawabe *et al.*, 1992; Sawabe *et al.*, 1997)
Marine bacterium ATCC 433367	Extracellular	PolyG	Exo – and endolytic	(Brown & Preston, 1991; Preston *et al.*, 1985)

Source	Localisation	Substrate specificity	Action mode	Reference
Photobacterium sp. (marine bacterium ATCC 433367)	Periplasm (R)	PolyM, acetylated	Endolytic	(Malissard *et al.*, 1995; Malissard *et al.*, 1993)
Pseudomonas alginovora (strain X017)	Extracellular	PolyG	Endolytic	(Boyen *et al.*, 1990)
Fungi				
Asteromyces cruciatus	Intracellular	-	Endolytic	(Schaumann & Weide, 1990)
Corollospora intermedia	Extracellular	-		(Schaumann & Weide, 1990)
Dendryphiella salina	Extracellular	PolyM	Endolytic	(Shimokawa *et al.*, 1997)
		-	Endolytic, with some exolytic	(Schaumann & Weide, 1990)
Virus				
Chlorella virus	Intracellular (R)	CL2: putative M	-	(Suda *et al.*, 1999)

Not determined; CL2, alginate lyase coded by open reading frame 2.
R = recombinant expression and localisation in *E. coli.*

Table 1.3: Characterisation of the reported alginate lyases.

Enzyme	Organism	Optimal pH	Optimal temperature (ºC)	Molecular mass (kDa)	Reference
AlgH-I	*Marinimicrobium koreense* H1	10	45	61.3	(Yan *et al.*, 2019)
AlyA	*Isoptericola halotolerans* NJ-05	7.5	55	25.32	(Zhu *et al.*, 2018)
AlgNJU-03	*Vibrio* sp. NJU-03	7.0	30	48.12	(Zhu *et al.*, 2018)
AlyH1	*Vibrio furnissii* H1	7.5	40	35.8	(Zhu *et al.*, 2018)
AlgMsp	Microbulbifer sp. 6532A	8.0	50	28	(Wang *et al.*, 2019)
AlyL1	*Agarivorans* sp. L11	8.6	40	36.36	(Li *et al.*, 2015)
Oal17A	*Vibrio* sp. W13	8.0	30	54.12	(Yu *et al.*, 2018)
AlgL	*Sphingomonas* sp. MJ-3	6.5	50	78	(Park *et al.*, 2014)
AlyM	*Microbulbifer* sp. Q7	7.0	55	63	(Yang *et al.*, 2018)
ALW1	*Microbulbifer* sp. ALW1	7.0	45	26	(Wang *et al.*, 2019)

1.3.1.3 Applications

Alginate lyases were normally used as an effective tool to degrade polysaccharides to oligosaccharide which showed many specific structures as well as various biological functionalities. For example, polyG lyase of *Streptomyces* sp. strain A5 produced alginate oligosaccharides that enhanced the germination and shoot elongation in plants (Kim *et al.*, 2011). The oligosaccharides exhibit many functions such as antitumor, anticoagulants, stimulating production of cytotoxic cytokines from human macrophages (Zhu & Yin, 2015).

Recently, the alginate lyases have become a useful agent in treatments of cystic fibrosis, in conjunction with antibiotics (Islan *et al.*, 2013; Islan *et al.*, 2014; Kim *et al.*, 2011). In addition, the alginate lyases have been employed in gene engineering and in the observation of *Fucus* cell wall development (Zhu & Yin, 2015). Therefore, alginate lyase is expected to be widely applied in therapeutics, medicine and biotechnology in the near future because of their biological activities.

1.3.2 Fucoidanase

1.3.2.1 Distribution and properties

The term “fucoidanase” was first noted in an article published in 1967, relating to the isolation of enzyme from the hepatopancreas of *Haliotus* sp. (Thanassi & Nakada, 1967). To date, fucoidanases have been observed in marine organisms: bacteria (Bakunina *et al.*, 2000; Descamps *et al.*, 2006; Silchenko *et al.*, 2008); invertebrates (Bilan *et al.*, 2005; Kitamura *et al.*, 1992; Kusaykin *et al.*, 2008) and some fungi (Rodriguez-Jasso *et al.*, 2010). Kusaykin *et al.* (2008) reported that the activities of fucoidanase produced by these microorganisms are low, whereas the

bacteria correlated with brown seaweed, sea cucumber and sea urchin are the best producers of fucoidanase (Bakunina *et al.*, 2002) Other efficient fucoidanase producers are salt marsh grass, sponges and molluscs (Daniel *et al.*, 2001; Holtkamp *et al.*, 2008).

There are differences in fucoidan structures obtained from diverse seaweed sources because different microorganisms can produce different types of fucoidanases with different actions. This can lead to the specific biological activities depending on the fucoidanase sources. For example, fucoidanases isolated from the marine bacterium *Pseudoalteromonas citrea* KMM 3296 are more specifically related to sulphated 1,3-α-L-fucans from brown seaweed *Laminaria cichorioides*, whereas fucoidanase of marine mollusk *Littorina kurila* prefer to catalyse the degradation of 1,3;1,4-α-L-fucans from *Fucus evanescens* (Kusaykin *et al.*, 2007).

Enzyme activity can depend on the environment. Fucoidanase from bacteria exhibited activity at alkaline pH. In contrasts, the enzymes from invertebrates are more active at acidic pH, but not in the case of the fucoidanases of marine mollusk *Littorina kurila* which has an optimum pH at 8.5 (Table 1.4). There are several studies of the influence of metal ions on the fucoidanase activity. Silchenko *et al.* (2013) demonstrated that fucoidanase isolated from the marine bacterium, *Formosa algae* strain KMM 3553, was activated by Mg^{2+}, Ca^{2+}, and Ba^{2+}, however, the presence of Cu^{2+} and Zn^{2+} caused inhibitory effects on its activity.

Figure 1.7: Enzymatic attack patterns of fucoidanase. Enzyme type 1: (1->4)-α-Lfucoidan endohydrolase, enzyme type 2: (1->3)-α-L-fucoidan endohydrolase, enzyme type 3: (1->3)-α-L-fucoidan exohydrolase. This figure has been reproduced from Ale & Meyer (2013)

Table 1.4: Properties of fucoidan degrading enzymes (Kusaykin *et al.*, 2016).

Source	Enzyme	Mode of action	T-optimum	pH-optimum	pH stability	Inhibitor	Activators	MW (kDa)	Reference
Bacteria									
Vibrio sp.No.5	E1 E2	Exo Exo	38 – 45 38 – 45	6.0 6.0	4.0 – 9.0 4.0 – 9.0	Hg^{2+}, Fe^{3+} Ag^{+}	Co^{2+}	39.5 68.0	(Furukawa *et al.*, 1992)
*Formosa algae*KMM3553T	E1	Endo	-	6.5 – 9.0		Cu^{2+}, Zn^{2+}	Mg^{2+}, Ca^{2+}, Ba^{2+}	96	(Silchenko *et al.*, 2013)
Alteramonas sp. SN-1009	E1	Endo	30 – 35	6.5 – 8.0		Cu^{2+}, Zn^{2+}	0.4M NaCl	100	(Sakai *et al.*, 2004)
Pseudoalteromonas citrea KMM 3296, KMM 3297, KMM 3298	E1	Endo	-	6.5 – 7.0	-	-	-	-	(Bakunina *et al.*, 2002)
Mariniflexile fucanivorans SW5T	E1	Endo	20 – 25	7.5	-	-	Ca^{2+}	105	(Descamps *et al.*, 2006)

Source	Enzyme	Mode of action	T-optimum	pH-optimum	pH stability	Inhibitor	Activators	MW (kDa)	Reference
Inverterbrates									
Haliotus sp.	E1	Endo	38	5.4	2.0 – 10.0	Hg^{2+},Mn^{2+} Ag^{+} (0.001M)	Mg^{2+} (0.01M)	100 - 200	(Thanassi & Nakada, 1967)
L. sitkana	E1 E2	Endo Endo	- -	5.4 8.5	-	- 0.2M NaCl	0.2M NaCl	- -	(Bilan *et al.*, 2005)
Lambis sp.	E1	Endo	45	5.0	-	Cu^{2+}, Zn^{2+}, Hg^{2+}	-	50	(Silchenko *et al.*, 2014)
Strongulocentrotus nudus	E1	Exo	45	3.0 – 4.0	2.0 – 5.0	-	-	130	(Sasaki *et al.*, 1996)
Fungi									
Dendryphiella arenaria TM94	E1	Endo	50	6.0	5.0 – 7.0	-	-	180	(Wu *et al.*, 2011)
Fusarium sp. LD8	E1	Endo	60	6.0	6.0	-	-	64	(Qianqian *et al.*, 2011)

1.3.2.2 Specificity of fucoidanase

The most important properties of fucoidanase are their specificity and the mode of their action. Fucoidanase can be classified into two groups including exo- and endo-fucoidanase. Exo-fucoidanase can be employed to cleave molecules of sulphated or nonsulphated fucose from the end of fucoidans. Endo-fucoidanase can hydrolysis the glycoside bonds inside a molecule of fucoidan and release various sizes of oligosaccharides. Almost all known fucoidanases have been found to be endo-type (Kusaykin *et al.*, 2016). The specificity of fucoidanases were usually determined by using various substrates with known structures. The mode of action of enzymes can be determined based on the structure of the enzymatic hydrolysis products of fucoidan. Furukawa *et al.* (1992) found three types of fucoidanases isolated from the marine bacterium *Vibrio* sp. N-5. All three fucoidanases showed an exo-type action and major products of enzymatic degradation with these enzymes were sulphated fucose and sulphated fucobioses (Table 1.5). Based on the literature review, in the same work, Descamps *et al.* (2006) have concluded that the fucoidanase from *Mariniflexile fucanivorans* SW5^{T} was specific to α-1→4 glycoside bonds in the backbone of fucoidan from brown algae *Pelvetia canaliculata* [3)-α-L-Fucp-(2,3OSO_3−)-1→4-α-L-Fucp-(2OSO_3−)-(1→], while fucoidanase from *Alteramonas* sp. SN-1009 was specific in hydrolysis of α-1→4 glycoside bonds when fucan sulphate was employed [3)-α-L-Fucp-(2,3OSO_3−)-1→3-α-L-Fucp-(2OSO_3−)-(1→] from *Kjellmaniella crassifolia* as the substrate (Takayama *et al.*, 2002).

To establish the specificity of fucoidanase extracted from the bacterium *Pseudoalteromonas citrea* KMM 3296, KMM 3297, KMM 3298, fucoidans from

Saccharina cichorioides (1→3-α-L-fucan) and *Fucus evanescens* (1→3; 1→4-α-L-fucan) were used as substrates. The cleavage of fucoidan from *F. evanescens* catalysed more efficiently with the enzyme from strain KMM 3296 than from *S. cichorioides*, in contract with the enzyme from strains KMM 3297 and KMM 3298. Oligosaccharides with structure [3)-α-LFucp-(2OSO_3−)-1→4-α-L-Fucp-(2,3OSO_3−)-(1→]$_n$ prevailed among the products of the enzymatic reaction. For this reason, fucoidanase from strain KMM 3296 was identified as an 1,3 α-L-fucoidan endohydrolase (Bakunina *et al.*, 2002).

Table 1.5: **Specificity of the fucoidanase**

Source	Enzyme	Mode of action, cleaved linkages	Substrate, structure	Products	Reference
Bacteria					
Vibrio sp.No.5	E1 E2 E3	Exo Exo Exo	*K. crassifolia* [3)-α-L-Fucp-(2OSO_3−)-1→3-α-L-Fucp-(2,3OSO_3−)-(1→] Branches: α-L-Fucp-1→2	Sulphated fucose and/or sulphated fucobiose	(Furukawa *et al.*, 1992)
Formosa algae KMM3553T	E1	Endo, α-1→4	*F. evanescens*	Sulphated fucooligosaccharides	(Silchenko *et al.*, 2013)
Alteramonas sp. SN-1009	E1	Endo, α-1→3	*K. crassifolia* [3)-α-L-Fucp-(2OSO_3−)-1→3-α-L-Fucp-(2,3OSO_3−)-(1→] Branches: α-L-Fucp-1→2	Sulphated fucooligosaccharides containing α-1→3 linked fucose residues	(Takayama *et al.*, 2002)
Pseudoalteromonas citrea KMM 3296,	E1	Endo, α-1→3	*Fucus evanescens*	Sulphated fucooligosaccharides containing	(Bakunina *et al.*, 2002)

KMM 3297, KMM 3298			[3)-α-L-Fucp-(2,4OSO_3^-)-1→4-α-L-Fucp-(2OSO_3^-)-(1→]; *Saccharina cichorioides* [3)-α-L-Fucp-(2,4OSO_3^-)-1→3-α-L-Fucp-(2,4OSO_3^-)-(1→]	α-1→3, α-1→4-linked fucose residues	
Mariniflexile fucanivorans SW5T	E1	Endo, α-1→4	*P. canaliculata* [3)-α-L-Fucp-(2OSO_3^-)-1→4-α-L-Fucp-(2,3OSO_3^-)-(1→]	Sulphated fucooligosaccharides containing α-1→3, α-1→4-linked fucose residues	(Descamps *et al.*, 2006)
Invertebrates					
Haliotus sp.	E1	Endo	*Fucus gardneri*	Fucose and fucooligosaccharides	(Thanassi & Nakada, 1967)
L. sitkana	E1 E2	Endo, α-1→3	*Fucus distichus* [3)-α-L-Fucp-(2,4OSO_3^-)-1→4-α-L-Fucp-(2,3OSO_3^-)-(1→]	Sulphated fucooligosaccharides containing α-1→3, α-1→4-linked fucose residues	(Bilan *et al.*, 2005)

Lambis sp.	E1	Endo, α-1→4	*F. evanescens*	Sulphated fucooligosaccharides containing α-1→3, α-1→4-linked fucose residues	(Silchenko *et al.*, 2014)
Strongulocentrotus nudus	E1	Exo, α-1→2	2-sulfo-α-L-fucopyranosyl-(1→2) pyridylaminated fucose	2-sulfo-α-L-fucopiranose	(Sasaki *et al.*, 1996)
Fungi					
Dendryphiella arenaria TM94	E1	Endo	*Fucus vesiculosus,* *Laminaria digitata*	Oligosaccharides	(Wu *et al.*, 2011)
Fusarium sp. LD8	E1	Endo	*F. vesiculosus*	Oligosaccharides	(Qianqian *et al.*, 2011)

1.4 Enzymes from actinobacteria

1.4.1 Actinobacteria

Actinobacteria are gram positive bacteria , with a high guanine (G) plus cytosine (C) ratio in their DNA (> 55%) (Goodfellow & Williams, 1983). Actinobacteria are considered to be one of the major groups of soil bacteria and have also been isolated from a variety of marine samples. One of the largest genera of actinobacteria is *Streptomyces*, which have many important and interesting features (Maleki & Mashinchian, 2011). *Streptomyces* have been considered as valuable producers of antibiotics and other useful compounds in therapeutics and as producers of vital industrial enzymes (Bizuye *et al.*, 2013; Genilloud, 2017). Some other important genera of filamentous actinobacteria are *Micromonospora,* and *Nocardia* which can produce various types of secondary metabolites (Salami, 2004). Marine actinomyces have been recognised as a source of new anticancer agents and antibiotics with novel structures and properties (Khalifa *et al.*, 2019; Subramani & Sipkema, 2019).

1.4.2 Enzyme production from actinobacteria

Among microorganisms, actinobacteria are considered a key source for the production of industrially important enzymes (Kafilzadeh *et al.*, 2012; Kumar *et al.*, 2012; Suneetha & Khan, 2011). Previously, various studies have shown that actinobacteria is the largest group which has the ability to produce novel secondary metabolites, especially antibiotics and industrial enzymes including gelatinase, cellulase, chitinase, lipase and caseinase (Bredholt *et al.*, 2008; Gulve & Deshmukh, 2012; Kumar *et al.*, 2012; Ramesh & Mathivanan, 2009). In addition, they are also

a vital source for the discovery of new biological products. For example, amylase is one of the most commonly used enzymes in starch industries. *Streptomyces, Bacillus, Pseudomonas, Micrococcus, Arthrobacter, Proteus, Escherichia,* and *Serratia* are the most important bacterial genera that produce amylase (Shafiei *et al.*, 2011). *Streptomyces* spp. have been explored as promising sources for the production of amylase (Hogue *et al.*, 2006; Kafilzadeh *et al.*, 2012; Selvam, 2011; Yassien & Asfour, 2011). In a study by Ragunathan and Padhmadas (2013), *Streptomyces* sp. PDS1 was reported to be a valuable candidate for amylase production. Amylase production had a maximum enzyme activity on 3rd day of incubation (56 U/mL). The optimum pH and temperature were 7.0 and 40^{o}C, respectively. The molecular weight of the partially purified enzyme was found to be 44 kDa. In another study, marine actinobacteria that had a strong ability to produce industrial amylase have been discovered by Kafilzadeh *et al.* (2012). This has a great significance within applications in biotechnology including food, fermentation, textile and paper industries.

It is widely accepted that actinobacteria will provide not only a valuable resource for novel enzyme products but also antimicrobial agents. For instance, L-glutaminase enzyme has attracted significant attention due to its potential application in medicine as an anticancer agent (Nandakumar *et al.*, 2003). Krishnakumar *et al.* (2011) had studied *Streptomyces* strain SBU1 that showed encouraging L-glutaminase activity. The highest L-glutaminase activity (18.0 U/ml) was observed when the enzyme was extracted from a medium containing 2% NaCl (w/v) and 1% malt extract (w/v) as nitrogen source, 1% glucose (w/v) as carbon source. The culture conditions by strain SBU1 were optimised at the pH of 9.0 after incubation for 96 hours at 30°C. In another study, Abdallah *et al.* (2013) optimised the conditions for the purification of

glutaminase from *Streptomyces avermitilis. Streptomyces avermitilis* was inoculated in mineral salt glutamine (MSG) medium including 1.0 g/L KH_2PO_4; 0.5 g/L $MgSO_4$; 0.1 g/L $CaCl_2$; 0.1 g/L $NaNO_3$; 0.1 g/L tri sodium citrate; 25 g/L NaCl; 10 g/L glucose. After 72 hours incubation at 28^{o}C in a rotary shaker at 120rpm, the inoculum was transferred into MSG production medium with phenol red (0.012%) at pH 7. The maximum yield of L-glutaminase production was obtained at inoculum size of 5ml/100ml (v/v) media, 3% NaCl, pH 8, temperature 28^{o}C after 5 days of incubation. Sodium nitrate and glucose were found as the best nitrogen and carbon sources, respectively. The partially purified enzymes were observed to have maximum activity at pH 7 and 8, and at the temperature of 30^{o}C. L-glutaminase from *Streptomyces avermitilis* was enhanced by introducing $MgSO_4$ and NaCl as mineral salts and was highly stable in the presence of 4% NaCl.

L-glutaminase enzyme plays a significant role in medical applications as an anticancer agent (Nandakumar *et al.*, 2003). It has been used for anti-lymphocytic leukemia 8, 9, 10. L-glutaminase produced by *Streptomyces canarius* FR (KC460654) was tested for the anticancer activity in five types of human cancer cell lines by using MTT assay. The results showed that Hep-G2 cell and HeLa cell were affected significantly by L-glutaminase. A reasonable cytotoxic effect of L-glutaminase was demonstrated in HCT-116 cell and RAW 264.7 cell (Reda, 2015). L-glutaminase from *Pseudomona*s sp. 7A and *Aspergillus niger* were reported for their activity against HIV virus and anti-tumor, respectively (Sunil *et al.*, 2014; Roberts *et al.*, 2001). L-glutaminase is also used as biosensors to monitor the glutamine levels in mammalian and hybridoma cells, so that the cancer cell can be detected (Sarada, 2013). For the requirement of commercial production of L-

glutaminase enzyme, marine actinobacteria have been explored to optimize the culture conditions for large scale production.

Additionally, actinobacteria and particularly *Streptomyces* spp. are considered as good sources of chitinase production. Several *Streptomyces* spp. such as *S. thermoviolaceus, Streptomyces plicatus*, *S. virdificans, S. lividans*, *S. aureofaciens*, *S. halstedii*, *S. diasitapiticus*, and *S. griseus* have been studied for identifying chitinolytic enzymes (Joo, 2005; Narayana & Vijayalakshmi, 2009; Taechowisan *et al.*, 2003). Narayana and Vijayalakshmi (2009) reported on chitinase production from a terrestrial *Streptomyces albidoflavus* ANU 6277 isolated from laterite soils of Acharya Nagarjuna University. This strain was inoculated in chitin yeast extract-salts (CYS) medium including 5.0 g/L chitin, 0.5 g/L yeast extract, 2.0 g/L K_2HPO_4, 1.0 g/L $MgSO_4.7H_2O$, and 0.1 g/L $FeSO_4.7H_2O$. The optimum culture conditions in CYS medium were studied for maximum chitinase production including chitin concentration, carbon and nitrogen sources, incubation time, pH and temperature. The results showed that strain ANU 6277 produced maximum chitinase when 1% chitin was added into CYS medium and was incubated for 60 hours. The chitinase reached its highest activity with pH 6 at 35^{o}C of the culture medium. Additionally, starch and yeast extract were proven as a good carbon and nitrogen source for chitinase production to enhance a chitinase yield. Chitinase of strain ANU 6277 purified by Sephadex G-100 displayed an obvious protein band around 45 kDa by running SDS-PAGE. This enzyme was reported to exhibit antifungal activity against a phytopathogenic mold *F. udum.* Therefore, it may be utilised as a biocontrol agent for wilt disease treatment.

In another study of Cao *et al.* (2007) isolated alginate lyase produced by Streptomyces strain A5 from banana rhizosphere and found that the alginate oligomers produced by the alginate lyase promoted root growth of banana plantlets. The extracted alginate lyase has a molecular mass of 32 kDa. The optimum temperature and pH were 37°C and 7.5, respectively. The Km value of the alginate lyase was 0.13 mg/mL with sodium alginate as the substrate. Additionally, alginate lyase from marine bacteria including *Streptomyces* sp. ALG-5 and *Streptomyces* sp. M3 were cloned and transformed into *E. coli* BL21 (Kim *et al.*, 2009; Kim, 2010). They were characterized to be PolyG specific lyases. The molecular weights of ALG-5 and M3 lyase were 28.2 kDa and 28.5 kDa, respectively. ALG-5 and M3 lyase showed the highest activity at pH 8 and 9, the temperature of 50^{o}C and 60^{o}C, respectively. They degraded alginate and produced di-, tri-, tetra- and pentasaccharides as the main products. In recent years, fucoidanase produced by marine actinobacteria has gained attention for the medical utilisation of fucoidans and the products of their degradation. Manivasagan and Oh (2015) had studied the production of unique fucoidanase used in the green synthesis of gold nanoparticles by *Streptomyces* sp. and its cytotoxic effect on HeLa cells. This can potentially have wide applications in anticancer therapy in modern medicine.

All of these studies indicate that actinobacteria are a superior source of bioactive compounds and prolific producers of enzymes that have a wide range of applications in the food, medical and agricultural industries.

1.5 Isolation, purification and characterisation of enzymes

1.5.1 Cultivation of microorganism for producing polysaccharide-degrading enzymes

Marine microorganisms isolated from seaweed, sea cucumber and sea mud have been considered as good sources for polysaccharide-degrading enzymes. The correlation between the production of polysaccharide-degrading enzymes and culture conditions may differ among different organisms (Table 1.6). With the aim of increased enzyme activity, the microorganism culture conditions, especially the medium and time conditions should be optimized to get the highest activity of polysaccharide-degrading enzymes.

Table 1.6: The culture conditions of different microorganisms produced polysaccharide-degrading enzymes.

Strains	Enzymes	Cultivating medium (All w/v, per litre)	Opt pH	Opt temperature (°C)	Cultivating time (days)	References
Gracilibacillus sp. A7 (from seaweed waste)	alginate lyase	0.5 g polypeptone, 0.3 g yeast extract, 5 g sodium alginate, 2 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5g NaCl	8.5 – 9.5	30	2	(Tang *et al.*, 2009)
Vibrio sp. QY105 (from sea mud)	alginate lyase AlyV5	3 g sodium alginate, 3 g KH_2PO_4, 7g $K_2HPO_4.3H_2O$, 2 g $(NH_4)_2SO_4$, 30 g NaCl, 0.05 g $FeSO_4.7H_2O$, 0.01 g $MgSO4.7H_2O$	7.0	25	4	(Wang *et al.*, 2013)
Microbulbifer sp. ALW1(from rotten brown seaweed)	alginate lyase	5 g sodium alginate, 30 g NaCl, 5 g $(NH_4)2SO_4$, 2 g K_2HPO_4, 1 g $MgSO_4 \cdot 7H_2O$, 1 g $FeSO_4 \cdot 7H_2O$	7.5	25	2	(Zhu *et al.*, 2016)
Vibrio furnissii H1(from rotten seaweed)	alginate lyase	6.0 g sodium alginate, 5.0 g tryptone, 2.5 g yeast extract, 25.0 g NaCl, 0.25 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.05 g $FeSO_4 \cdot 7H_2O$	7.5	28	2	(Zhu *et al.*, 2018)
Pseudoalteromonas sp.M3HP2 *(*from sea cucumber *A. japonicus)*	fucoidanase	5g Difco bactopeptone, 2g Difco yeast extract, 1 g fucoidan, 0.1 g K_2HPO_4, 0.05 g $MgSO_4$, 100% seawater	7.5 – 7.8	28	2	(Bakunina *et al.*, 2000)
Flavobacteriaceae SW5	fucoidanase	Zobell-Fucan medium (5g bacto pepton, 1g yeast extract, 2 – 5 g Fucan, 800ml seawater and 200ml distilled water	7.8	22	5	(Colin *et al.*, 2006)

Strains	Enzymes	Cultivating medium (All w/v, per litre)	Opt pH	Opt temperature (°C)	Cultivating time (days)	References
Formosa algae strain KMM 3553 (from brown algae *F. evanescens*)	fucoidanase	5 g Bacto peptone, 1 g Bacto yeast extract, 1 g glucose, 0.2 KH_2PO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, 50% natural seawater and 50% distilled water	7.8	28	2	(Silchenko *et al.*, 2013)
Streptomyces sp. (from marine sediment samples)	fucoidanase	5 g wheat bran, 1 g kelp powder, 0.5 g glucose, 0.05 g $NaNO_3$, 0.05 g $MgSO_4 \cdot 7H_2O$, 1 g NaCl, and 100% seawater	7.5	28	7	(Manivasagan & Oh, 2015)

1.5.2 Concentration of enzymes

Concentration is an essential step to recover enzymes since enzymes contained in solution have very low concentration along with thousands of other compounds. Extracellular enzymes rarely exceed a few grams per litre. The microbial enzymes are usually unstable and susceptible because of the attraction of microorganisms, since they cannot be stored as crude supernatant solutions. Therefore, concentration is an initial necessary step to remove a large volume of water and then further purification steps can be applied.

Precipitation is one of the simplest methods used for the concentration of proteins. The most common methods of protein enrichment rely on selective precipitation using acetone, trichloroacetic acid, ethanol, isopropanol, diethylether, chloroform/ methanol, and polyethylene glycol (Chen *et al.*, 2005; Zellner *et al.*, 2005). It is

widely known that among the different precipitates the most common is ammonium sulphate, which causes protein destabilization (Bodzon-Kulakowska *et al.*, 2007). The high amounts of ammonium sulphate are added into a protein solution leading to initiate an increase of protein interactions and then protein can be aggregated and finally precipitated. This is known as a salt removing process and different types of protein need different salt concentrations for its precipitation, allowing separation of selective proteins. An *et al.* (2009) reported that alginate lyase obtained from newly isolated *Flavobacterium* strain LXA was partially purified by 45% ammonium sulphate precipitation to use for algino-oligosaccharide production. Dialysis is also ultilised to concentrate protein solutions. The process of removal of salt molecules from the inside of dialysis bags would be achieved by using a dialysis membrane immersed in a selected buffer (Andrew *et al.*, 2001).

Freeze-drying has also been considered as another method for protein concentration (Wasserman, 1995). Freeze drying can provide a stable product which can be stored easily if further processing must occur on a staggered time basis. It is a convenient method to prepare high purity protein and is widely used in application.

1.5.3 Purification of enzymes

To purify enzymes, various techniques are used based on the chemical and physical properties of the protein.

Dialysis is an extensively used and simple method to remove salts from protein solutions (Berg *et al.*, 2002). Chromatography has been applied in terms of purification and detection of biological molecules. The principle separation of

chromatography is to achieve distribution of separated molecules between a stationary phase and a mobile phase. The classification of chromatographic methods is dependent on the physical shape of stationary phases, the nature of mobile phases and/or stationary phases, and mechanism of separations. Moreover, the classification of chromatographic methods is also based on mechanisms of separation, including adsorption chromatography, partition chromatography, size-exclusion chromatography, ion-exchange chromatography and affinity chromatography (Wilson, 2010). Column chromatography including high performance liquid chromatography (HPLC) and fast protein liquid chromatography (FPLC) are extensively used for enzyme purification. These methods are usually used to separate protein according to their charge, size, or binding affinity. The separated proteins are eluted from the column, indicated by their peaks which can then be detected by absorption spectrometry and spectrofluorometry.

Ion-exchange chromatography has been widely used for enzyme purification due to commercial significance (Aguilar *et al.*, 2006; Liu & Xia, 2006). In the ion-exchange chromatographic technique, the proteins were separated according to magnitude of net electric charge and this technique is classified into two types including cation-exchange chromatography and anion-exchange chromatography. The material packed in the column is an ion exchange resin of which there are two types - a cation and an anion exchanger (Karlsson & Hirsh, 2011). For example, DEAE-cellulose is an anion exchanger possessing positively charged groups and DM-cellulose is a cation exchanger having negatively charged groups. For the purification of extracellular alginate lyase produced from *Streptomyces* strain A5 by Cao *et al.* (2007), crude enzyme supernatant after centrifugation was loaded into DEAE-cellulose column (2.5 - 40 cm), primarily equilibrated with phosphate buffer (pH 7.0)

and finally eluted with a linear gradient of NaCl (0 - 2.0 M) in the same buffer. The active fractions were concentrated and used as purified enzymes. To enhance the purity of enzyme, hydrophobic interaction chromatography, gel filtration chromatography (size exclusion chromatography) or affinity chromatography can be used in another step of purification procedures.

1.5.4 Characterisation of enzymes

SDS-PAGE is generally used to estimate purity and molecular weight of proteins (Bakare *et al.*, 2005). The principle of electrophoresis is based on charged proteins migrating within an electric field. Electrophoresis of proteins is developed in a gel medium using polyacrylamide. The principle of this method is that sodium dodecyl sulphate (SDS) binds to most proteins with amount nearly proportional to the molecular weight of the proteins. Thus, each protein will have similar charge-to-mass ratio and will migrate through the gel in relativity to its molecular weight. The buffers used in this method include stacking gel and separating gel. There are differences in composition, concentration and pH among electrolyte buffers, stacking buffers and separating buffers. The proteins separated on a polyacrylamide gel can be visualised by different types of staining depending on the target enzyme. Staining with the dye Coomassie Brilliant Blue R250 is the most broadly used because it is convenient, low cost and effective for visualising proteins. However, silver staining and other dyes are also available for special purposes (Holtzhauer, 2006).

To characterise enzymes of interest from microorganisms, structures of degradation products are analysed by chromatographic methods. Thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography

(GC) are suitable for identifying known chemical structures of hydrolysis products of enzymes. The enzyme products of unknown structure can be analysed by spectroscopic methods in which nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are most useful. Both MS and NMR can be used for identifying target proteins and providing spectroscopic information for protein components (Schneider, 2010).

NMR spectroscopy is one of the most informative techniques for identifying the structure of enzymes. The special advantages of NMR is that it has the ability to distinguish a variety of types of isomeric compounds such as diastereomers and stereoisomers, which sometimes are difficult to discriminate by mass spectrometric methods. Moreover, NMR is non-destructive, by which, the sample is still available post NMR measurements and can be used for other purposes such as mass spectrometric analysis or other analytical methods. Therefore, NMR spectroscopy is being widely used for identifying hydrolysis products of enzymes. In the research of Silchenko *et al.* (2017), identifying the structure of products that hydrolyse by fucoidanase FFA2 from marine bacterium Formosa algae, 1H, 13C spectra and 2D spectra (COSY, TOCSY, HSQC, HMBC) were used for the samples of polysaccharides and oligosaccharides in D_2O and acetone was used as the internal standard.

1.6 Research plan

1.6.1 Hypothesis:

Seaweed is a potential source of actinobacteria that produce novel polysaccharide-degrading enzymes.

1.6.2 Aims:

The objectives of the project are:

1. To isolate actinobacteria from decomposing seaweed collected at Rivoli Bay, Beachport, South Australia.

2. To screen actinobacteria that produce polysaccharide-degrading enzymes including alginate lyase and fucoidanase.

3. To purify the enzymes by using anion exchange and size exclusion chromatography.

4. To characterize the purified enzymes (molecular weight, substrate specificity, mode of action, optimum pH and temperature, effect of NaCl and metal ions).

5. To identify amino acid sequences and gene sequences encode polysaccharide-degrading enzymes by MS/MS.

6. To clone and express the enzyme into *E. coli* and produce it as a recombinant protein.

7. To apply the target enzymes and evaluate their function in degrading commercial alginate.

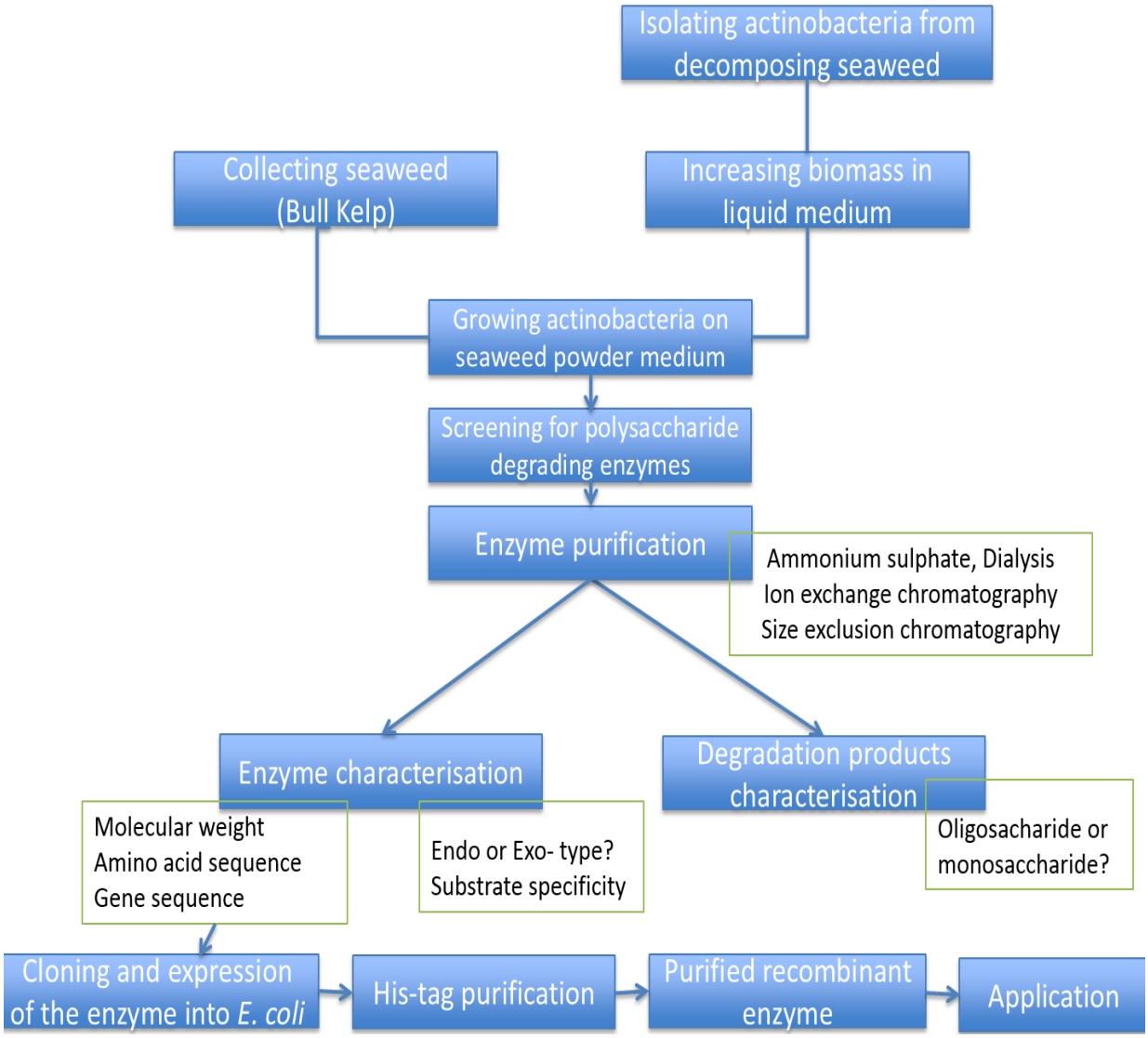


Figure 1.8: Flow diagram of research outline.

CHAPTER 2: MATERIALS AND GENERAL METHODS

2.1 Collection of seaweed samples

The seaweed used as a substrate in this study was *Durvillaea potatorum* (Bull Kelp), which is a brown seaweed. Bull Kelp was collected from the beach cast wrack at Rivoli Bay, Beachport, South Australia (Figure 2.1) in early 2017, under a licence given to Australian Kelp Products. The beach cast wrack that collects along 100 meters of coastline has an estimated 100 types of seaweed and can weigh up to 4 tonnes (Figure 2.2). The collected seaweed was sun dried at Beachport before being sent to Flinders University (Figure 2.2). The samples were kept in the undercroft of the Health Science building at room temperature until used.

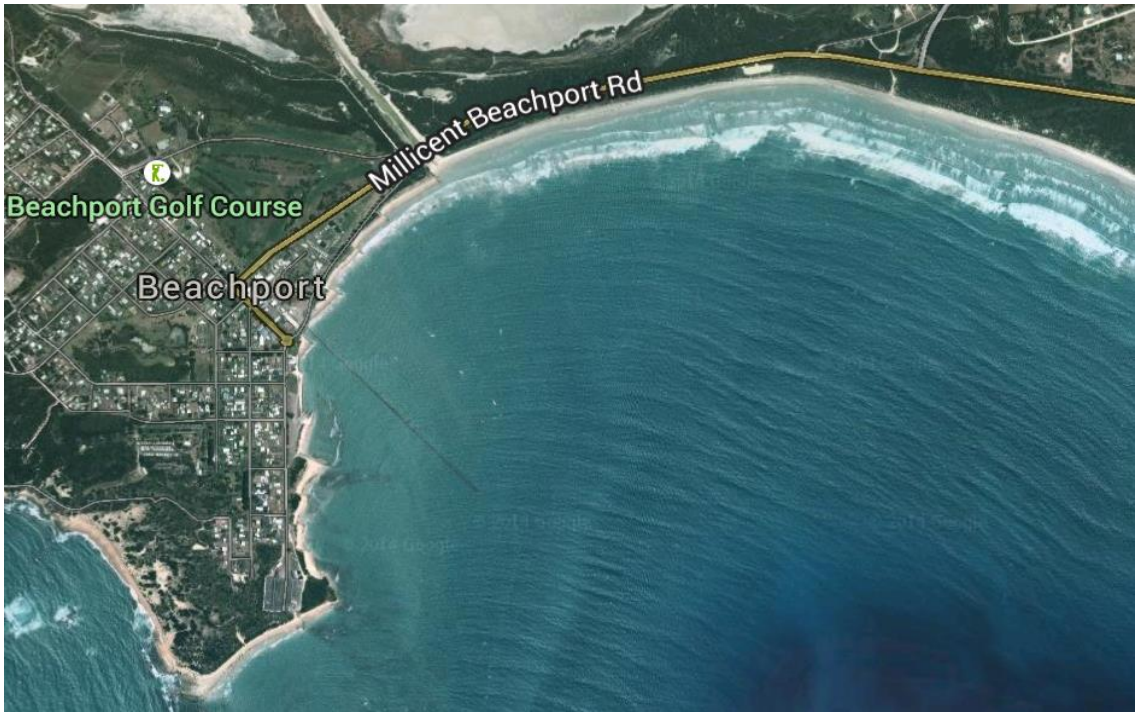


Figure 2.1: Location of Beachport in the southeast of South Australia.

Figure 2.2: Collection of seaweed at Rivoli Bay, Beachport, South Australia.

2.2 Isolation of actinobacteria from decomposing seaweed

2.2.1 Collection of seaweed for isolation of actinobacteria

Decomposing seaweed which included different kinds of brown, red and green seaweed were collected at Rivoli Bay, Beachport, South Australia (Figure 2.3) in July 2017. They were kept in plastic bags at 27^{o}C to allow further growth of microorganisms and processed within 7 days.

Figure 2.3: Decomposing seaweed from Rivoli Bay, Beachport, South Australia.

2.2.2 Isolation of seaweed-degrading microorganisms

The isolation media were:

- Humic acid vitamin B agar (HVA) (Appendix A1.1)
- Glycerol asparagine agar (GAA) (Appendix A1.2)
- Starch yeast peptone agar (SYP) (Appendix A1.3)

All media were autoclaved for 15 minutes at 121^{o}C and were supplemented with 50% seawater.

Decomposing seaweed samples (1 g/plate) were plated onto three isolation media in triplicate for each medium. The plates were kept in plastic boxes which were sealed by lids and wet paper towels were put at the bottom of plastic boxes to maintain the moisture during the incubation period. The plates were incubated at a temperature of 27^{o}C for 6 weeks. The number of colonies and their incubation time were recorded and any actinobacteria cultures removed and sub-cultured every week.

2.2.3 Purification of isolates

All single colonies were picked from isolation media and purified by streaking onto half-strength potato dextrose agar (HPDA) (Appendix A2). The actinobacteria colonies were transferred to fresh agar plates every month for sub-culture and the spores were kept in sterile glycerol at -20^{o}C for further study.

2.3 Screening actinobacteria that produce alginate lyase and fucoidanase

2.3.1 Actinobacterial cultivation

Two full loops of spore mass of isolated actinobacteria grown on HPDA plates for at least 7 days were inoculated into 50 ml of IM22 inoculum medium (Appendix A3) in 250 ml Erlenmeyer flasks and then cultured at 27^{o}C with shaking at 150 rpm. After three days, the inoculum was transferred to 50 ml production medium in 250 ml Erlenmeyer flasks at 5% (v/v). The production medium were prepared by adding 2% seaweed powder into 5 g/L peptone, 1 g/L yeast extract, 1 g/L glucose, 10 g/L NaCl, 2 g/L K_2HPO_4, 0.2 g/L $MgSO_4.7H_2O$ and 5 g/L NH_4Cl. The medium was sterilised by autoclaving at 121^{o}C for 15 min. After inoculation, the cultures in 50 ml medium per 250 ml Erlenmeyer flasks were incubated at 27^{o}C on a rotary shaker at 150 rpm for 7 days.

2.3.2 Enzyme assay (DNS assay)

The activities of polysaccharide-degrading enzymes were determined by measuring the amount of reducing sugar produced by enzymes present in the sample, following the method of Nelson (1994). The substrates used include 0.5% sodium alginate

(Sigma-Aldrich) and 0.5% fucoidan (Marinova, Australia) in 0.02M Tris-HCl buffer (pH 7.5). A reaction solution containing 0.5 ml crude enzyme and 0.5 ml substrate was incubated in a water bath at 37^{o}C for two hours. After that, 1 ml of 3-5-dinitrosalicylic acid reagent was added to stop the reaction between enzyme and substrate (Miller, 1959) and then the sample was boiled for 5 minutes. The colour of the sample was changed by the reaction between DNS reagent and any reducing sugar produced by the enzyme (Figure 2.4). The absorbance was measured in an UV Spectrometer at wavelength of 540 nm. The concentration of released reducing sugars was determined by using D-glucose and fucose as standards for alginate lyase and fucoidanase, respectively. A unit of enzyme activity is defined as the amount of enzyme which is required to produce 1 µmol of reducing sugars per min. The results were statistically analysed by One-way ANOVA on SPSS.

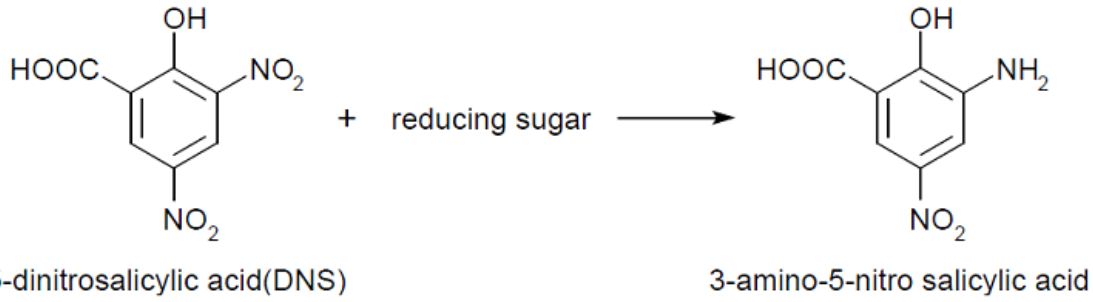


Figure 2.4: Reaction between DNS reagent and reducing sugar.

The enzyme activity was calculated following the formula below:

$$\text{Enzyme activity (U/ml)} = \frac{(\mu\text{moles of reducing sugar released}) \times V_1}{V_2 \times T}$$

V_1: Total volume of reaction mixture (ml)

V_2: Volume of enzyme in reaction mixture (ml)

T: Incubation time (minutes)

2.3.3 Protein quantification assay (BCA assay)

Protein concentration was analysed using a Pierce BCA Protein Assay Kit (Thermo Scientific, USA) as per manufacturer's instruction. Protein standard (bovine serum albumin- BSA) was prepared from 1.41 mg/mL stock solution (Appendix B2).

The BCA working reagent (WR) was prepared by using the formula to calculate the total volume of required WR as follow:

(≠ standards + ≠ samples) x (≠ replicates) x (volume of WR per sample) = total volume of required WR

The working reagent was prepared by adding BCA reagent A and reagent B with a ratio of 50:1 (reagent A:B) and then mixing with a vortex mixer. Ten microlitres of standard and each sample were then pipetted into 96 well plates (Costar, US) and 200 µl of the WR was added to each well and the plate was mixed on a shaker for 1 minute. The plate was covered with a lid and incubated at 37^{o}C for 30 minutes. Then, the plate was cooled down to room temperature and placed into a plate reader (GloMax Explorer, Promega) to measure the absorbance at 560 nm. The unknown protein concentrations were calculated based on the protein standard curve.

2.4 Optimisation of culture conditions

2.4.1 Incubation time

Selected cultures were inoculated into 50 ml IM22 inoculum medium in 250 ml Erlenmeyer flasks and then cultured at 27^{o}C with shaking at 150 rpm. After three days, 5 ml of the inoculum were transferred into 100 ml production medium in 500 ml Erlenmeyer flasks and then incubated at 27^{o}C from day 0 to day 10 with shaking

at 150 rpm. 5ml samples were withdrawn every day for testing enzyme activity by the DNS assay to find the time of maximum enzyme production.

2.4.2 Composition of culture medium

The medium components that significantly affected polysaccharide-degrading enzyme production were optimized using Response Surface Methodology (RSM). Three independent variables: peptone (g/L), yeast extract (g/L), NaCl (%) and the range of these variables were selected. Box – Behnken designs were used with three factors (peptone, yeast extract and NaCl) and three levels (-1, 0, 1) for the RSM.

2.5 Enzyme purification

2.5.1 Ammonium sulphate precipitation

The crude enzymes which were collected from broth cultures after the centrifugation at 10,000 g for 20 min at 4^{o}C were precipitated by ammonium sulphate at a concentration of 20%, 40%, 50%, and 60% at pH 7.5 and kept overnight at 4^{o}C. Then, the solutions were centrifuged at 10,000 g at 4^{o}C for 15 min. The precipitates were collected separately and dissolved in 20 mM Tris-HCl buffer.

2.5.2 Dialysis

The precipitates, containing the enzyme, dissolved in 20 mM Tris-HCl buffer, were dialyzed by using dialysis tubing cellulose membrane (14,000 molecular weight cut off, Sigma Aldrich) in 1 litre of the same buffer and then were left overnight at 4^{o}C

for desalting. After dialysis, the supernatants were used as partially purified enzymes for testing enzyme activity.

2.5.3 Ion exchange chromatography

The dialysed samples were loaded onto a Mono-Q™ column (5/50 GL, GE Healthcare) attached to FPLC machine (ÄKTA™ pure, USA). The mobile phase includes buffer A (20mM Tris-HCl pH 8.0) and buffer B (1M NaCl, 20mM Tris-HCl pH 8.0). The pump was run at a flow rate of 1 ml/min. The enzymes were eluted with a linear gradient of 0 – 1 M NaCl in the buffer B. The fractions obtained from anion exchange chromatography were evaluated for enzyme activity by the DNS assay and protein concentration by the BCA assay. The fractions possessing the highest specific enzyme activity were kept in the freezer at -20^{o}C before subjected for further purification.

2.5.4 Size exclusion chromatography

The purified fractions from anion exchange chromatography were loaded onto a Superdex™ 75 column (10/300 GL, GE Healthcare). The enzymes (1 ml) were loaded and eluted with 20 mM Tris HCl (pH 8.0) buffer containing 0.1 M NaCl at a flow rate of 0.5 ml/min. The fractions obtained from size exclusion column were evaluated for their activity and protein. The fractions of high specific enzyme activity were kept in the freezer at -20^{o}C.

2.6 Characterisation of purified enzymes

2.6.1 SDS-PAGE

The molecular weight of the purified enzymes was determined by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). A 4-20% Criterion™ TGX Stain-Free™ Precast Gels (10 or 18 wells, Bio-Rad) was used for running SDS-PAGE. Five microliters of Precious Plus Protein Unstained Standards (Bio-Rad) was used as a molecular mass marker. 5µl of 4 x loading buffer (Appendix D1.2) was added to 15µl of sample, then the mixture was boiled at 95^{o}C for two minutes, centrifuged briefly and loaded onto the gel. The gel was electrophoresed at 300V for approximately 20 minutes using criterion tank (Bio-Rad) containing 1 x running buffer (Appendix D1.1). After electrophoresis, the gel was placed on Stain Free Tray and image of gel was automatically taken by Gel Doc ™ EZ imager to visualise protein bands.

2.6.2 Amino acid sequencing of target enzymes

The amino acid sequences of polysaccharide-degrading enzymes were identified by using 5600+ qTOF mass spectrometry (AB Sciex, Framingham, MA, USA) fitted with an Ekspert nano LC 415 high performance liquid chromatography (HPLC) (Eksigent, AB Sciex). The results were analysed by Pilot Protein software (version 4.5).

2.6.3 Effect of various compounds on polysaccharide-degrading enzymes activity

- *Determination of the optimal pH of polysaccharide- degrading enzymes*: The reaction solution containing purified enzyme (100 µl) and substrate (500 µl) (0.5% sodium alginate toward alginate lyase) in 0.05M Tris- HCl buffer with a range of pH values (4.5, 5.5, 6.5, 7.5, 8.5, 9.5 and 10.5) were incubated for 2 hours at 37^{o}C. The enzyme activity was measured by the DNS assay following the Nelson method.

- *Determination of the optimal temperature of polysaccharide- degrading enzymes*: The purified enzyme was incubated with the substrate (0.5% sodium alginate toward alginate lyase) in 0.05M Tris- HCl buffer at different temperature of 15^{o}C, 25^{o}C, 35^{o}C, 45^{o}C, 65^{o}C and 75^{o}C for 2 hours. The enzyme activity was measured by the DNS assay.

- *Determination of salt tolerant of polysaccharide- degrading enzymes:* The purified enzymes were incubated with the substrate (0.5% sodium alginate toward alginate lyase) in 0.05M Tris- HCl buffer at different NaCl concentration of 0.2 M, 0.4 M, 0.6 M, 0.8 M and 1.0 M for 2 hours at 37^{o}C. The enzyme activity was measured by the DNS assay.

- *Influence of multivalent metal ions on polysaccharide- degrading enzymes:* The reaction mixture containing purified enzyme and substrate (0.5% sodium alginate toward alginate lyase) in 0.05M Tris- HCl buffer and 0.2 ml of 5 mM the appropriate salt ($CaCO_3$, $CoCl_2$, $CuSO_4$, $FeSO_4$, $FeCl_3$, $MgSO_4$, $MnCl_2$, $ZnCl_2$) were incubated for 2 hours at 37^{o}C. The enzyme activity was measured by the DNS assay.

2.6.4 Enzymes specificity

The substrate specificity was analysed using the DNS assay with three different substrates including sodium alginate, polymannuronic - PolyM, and polyguluronic – PolyG (Sigma-Aldrich). The enzymatic hydrolysis was monitored by electrospray ionisation mass spectrometry (ESI-MS) to indicate whether fucoidanase and alginate lysase were working as exo- or endo-type enzymes. Based on this, the structure of oligosaccharide degradation products can be explored.

2.7 Molecular cloning and expression of polysaccharide-degrading enzymes in *Escherichia coli* (*E. coli*)

To clone the alginate lyase genes into an expression vector, the restriction enzymes including *Nde*1 and *Sal*1-HF (High Fidelity) (New England BioLabs, US) were used. The gene sequences were optimised for *E. coli* expression by using Codon optimisation Tool from the IDT (Integrated DNA Technologies) website. The Gblock gene fragments were synthesised by IDT Company. The Gblock gene fragments were first ligated with pGEM-T easy vector (Promega, USA). Then, the secreted genes were ligated into pColdI expression vector (Takara, Japan). Briefly, recombinant *E. coli* containing pColdI/secreted gene plasmid was cultured in Luria-Bertani (LB) medium supplement with 100 µg/ml ampicillin and cloned protein was overexpressed by induction with 0.1 – 1M IPTG at 15^{o}C for 24 hours. The SDS-PAGE was used to analyse the overexpressed proteins. The recombinant protein containing $(His)_6$-tagged was purified by Ni^{2+} Sepharose affinity chromatography.

2.8 Screening alginate lyase genes of actinobacteria from different sources

The presence of the genes of alginate lyase was tested in actinobacteria isolated from a range of sources. This included 80 strains isolated from seaweed, 100 strains isolated from marine sponge, 100 strains isolated from plants, 100 strains isolated from soil were collected. Their DNA were extracted by using CTAB and 100 micron zirconium beads. And then alginate lyase genes were amplified by running PCR using primers that were designed for encoding alginate lyase genes. Finally, the PCR product were analysed by gel electrophoresis to establish a band of amplified alginate lyase gene.

2.8.1 DNA extraction

DNA of actinobacteria was extracted using CTAB (hexadecyltrimethyl ammonium bromide) extraction buffer (Doyle & Doyle, 1987). Actinobacteria were incubated on a sterilised cellophane strip on half-strength potato dextrose agar (HPDA) for 7 days at 27^{o}C for spore formation. Two full loops of actinobacteria cells were added in 500 µl of modified CTAB extraction buffer (pH 8.0) in sterile screw-capped microcentrifuge tubes. Half a gram of zirconium beads (100 micron) was added, vortexed briefly, then 500 µl phenol:chloroform:isoamylalcohol (25:24:1) was added and the tube was shaken in a bead-beating instrument for 5 minutes and then incubated for one hour at 56^{o}C in a water bath. After incubation, the tubes were centrifuged at 13,000 rpm for 5 minutes at 4^{o}C and then the aqueous top layer was transferred to new sterile 1.5 ml Eppendorf tubes. Chloroform:isoamylalcohol (24:1) was added to the tubes at 500 µl each, mixed well, and centrifuged at 13,000 rpm

for 5 minutes at room temperature. The aqueous top layer was transferred to another sterile 1.5 ml Eppendorf tubes. Two volumes of PEG/NaCl were added to precipitate the solution and the tubes were incubated in the fridge for 2 hours. Afterwards, the tubes were centrifuged at 13,000 rpm for 15 minutes at 4^{o}C. The supernatant was poured gently without disturbing the DNA pellet. The DNA pellet was washed by using 70% ice-cold ethanol and then spun again at 13,000 rpm for 5 minutes at 4^{o}C. The supernatant was poured gently without disturbing the DNA pellet. Resulting the pellet was removed carefully using a micropipette. Finally, the pellet was resuspended in 50 µl free ARN/DNA water.

2.8.2 DNA qualification

A NanoDrop spectrophotometer (ND-8000 spectrophotometer) was used for DNA quantification. Firstly, the active pedestal was blanked by using 2 µl of water. Both upper and lower pedestals were gently wiped using a Kimwipe. Afterwards, the samples (2 µl) were applied to active pedestals and then the optical density was measured. Finally, 5 µL of water was applied to the pedestal, the lid had been lowered for 2 minutes and then the water was gently wiped off with a Kimwipe. This step was repeated twice to make sure the active pedestal was completely clean.

2.8.3 PCR amplification of the polysaccharide-degrading enzyme gene

PCR was carried out with the designed primers to amplify the alginate lyase gene in actinobacteria. The PCR was carried out in 10 µl reaction volumes with the following reagents.

Table 2.1: PCR master mix ingredients and volumes.

Component	Volume
Taq 2X master mix	5 µl
MgCl	0.2 µl
10µM forward primer	1 µl
10µM reverse primer	1 µl
DNA sample (concentration of 200ng)	1 µl
Nuclease free water	1.8 µl
Total	10 µl

Afterward, all PCR master mix ingredients (Table 2.1) were added into each PCR tube. The reaction mixture was mixed gently and briefly centrifuged. Finally, the PCR tubes were put into the PCR machine. PCR reactions conditions are given in Table 2.2.

Table 2.2: PCR conditions.

Step	Temperature	Time
Initial Denaturation	94°C	2 min
30 Cycles	94°C 50-65°C 72°C	60 seconds 60 seconds 2 minute
Final Extension	72°C	10 minutes
Hold	10°C	

2.8.4 Purification of PCR samples

UltraClean ® PCR Clean-Up Kit (MoBio) was used to purify the PCR samples. Five volumes (50 ul) of SpinBind were added to each PCR reaction. The PCR-SpinBind mixtures were mixed well by pipetting before transfer to a spin filter unit. They were centrifuged at a minimum 13,000 rpm for 30 seconds. The spin filter baskets were taken out and the flow-through liquid from the tubes was discarded. Afterwards, the spin filter baskets were replaced in the same tubes and 300 µl SpinClean™ Buffer was added to the spin filter. The tubes were centrifuged at a minimum of 13 000 rpm for 30 seconds. Similarly, the spin filter baskets were removed, and the flow-through liquid was discarded, then the baskets were replaced in the same tubes and were centrifuged at a minimum 13,000 rpm for 60 seconds to remove all traces of SpinClean™ buffer. The spin filter baskets were transferred to clean 2 ml collection tubes and 50 µl of Elution Buffer (10 mM Tris) solution added directly onto the centre of the white spin filter membrane. They were centrifuged at a minimum 13,000 rpm for 60 seconds. Finally, the spin filter baskets were taken out and then the tubes were sealed and stored at -20°C.

2.8.5 Agarose gel electrophoresis

The PCR samples were checked for quality by gel electrophoresis. A 1% agarose gel was prepared by dissolving 0.4 g agarose in 40 ml of 0.5X TBE buffer (Appendix D2.1) and then melting in the microwave. The gel solution was left to cool and then 4 µl of Gel Red (Biotium) was added. After mixing well, the gel solution was poured gently to avoid making bubbles into the gel mound. After the gel had set, 0.5X TBE was poured into the electrophoresis tank to submerge the gel. The 5 µl DNA amplification products were mixed with 2 µl of loading dye and loaded into the wells

along with 5 µl of a 25 bp – 1000 bp DNA ladder. The gel was run at 100 V for 40 min. Gels were stained with ethidium bromide and photographed under UV light.

ZCHAPTER 3: ISOLATION OF ACTINOBACTERIA FROM SEAWEED AND SCREENING FOR POLYSACCHARIDE-DEGRADING ENZYMES

3.1 Introduction

Actinobacteria are known as one of the major groups of soil bacteria that have also been isolated from a wide range of marine samples. One of the genera of actinobacteria that has the largest number of species identified to date is *Streptomyces*, which hold many important and intriguing features. Dhanasekaran (2012) said that “Enzymes, after antibiotics, are the most important products of *Streptomyces*”. Therefore, *Streptomyces* are not only valuable producers of antibiotics but also potential producers of industrially important enzymes such as amylase, cellulase, gelatinase, casein hydrolysate, chitinase and lipase (Bredholt *et al.*, 2008; Gulve & Deshmukh, 2012; Kumar *et al.*, 2012; Ramesh & Mathivanan, 2009). These enzymes can be applied generally in biotechnology and specifically in the nutrition and biomedical fields (Nawani *et al.*, 2013).

In recent years, marine actinobacteria have been reported to produce polysaccharide-degrading enzymes such as alginate lyase and fucoidanase (Zhang & Kim, 2010). There are many avenues for the exploitation of the treated alginate and fucoidan and their degradation products (Holtkamp *et al.*, 2008).

On the other hand, seaweed has been considered as a valuable natural source of bioactive compounds. They have high protein content and varying levels of carbohydrates. The carbohydrate content of brown seaweed is 30 – 50%, consisting mainly of alginate, fucoidan and laminarin (Sakatoku *et al.*, 2012). Therefore, seaweed is a good carbon source for the growth of microorganisms including actinobacteria.

In this chapter, actinobacteria were isolated from a mixture of different kinds of decomposing seaweed collected at Rivoli Bay, Beachport, South Australia by Australian Kelp Products and forwarded to us via our Joint Laboratory. Different nutrient media, with long incubation times, were applied for the isolation. Polysaccharide-degrading enzymes were extracted from the culture medium of isolated actinobacteria in which Bull Kelp powder, an abundant type of brown seaweed in South Australia, was used as a carbon supplement. They were screened for alginate lyase and fucoidanase. The enzyme activity was increased by the optimisation of nutrient components and incubation time.

3.2 Materials and Methods

3.2.1 Isolation of seaweed degrading actinobacteria

The methods of seaweed collection, isolation and purification of actinobacteria were explained in sections 2.1 and 2.2. The number of isolated strains and their incubation time were recorded every week for four weeks.

3.2.2 Screening actinobacteria that produce alginate lyase and fucoidanase

All strains of isolated actinobacteria were screened for the production of alginate lyase and fucoidanase. Bull Kelp powder of 2% was used as a carbon source to induce the production of alginate lyase and fucoidanase. All the strains were inoculated in IM22 seed medium which was used to inoculate in production medium containing 2% Bull Kelp powder as described in section 2.3.1.

After 7 days incubation, broth cultures of 50 ml in a 250 ml Ehrlenmeyer flask were centrifuged at 10,000 rpm for 20 min at 4^{o}C to remove the cells and other debris. The cell-free supernatants were collected after centrifugation and stored as crude enzymes at 4^{o}C. The enzyme activities against alginate and fucoidan were determined by DNS assay as explained in section 2.3.2.

3.2.3 Optimisation of culture conditions

3.2.3.1 Optimisation of culture medium

The medium components that significantly affected polysaccharide-degrading enzyme production were optimized using Response Surface Methodology (RMS). Three independent variables including peptone (g/L), yeast extract (g/L), NaCl (%) and the range of these variables were selected. Box – Behnken designs with three factors and three levels for the RMS are shown in Table 3.1.

Table 3.1: Maximum and minimum levels of the variables used in the Box-Behnken design.

Independent variables	Symbols	Code levels		
		-1	**0**	**1**
Peptone (g/L)	A	1	8	15
Yeast extract (g/L)	B	1	5.5	10
NaCl (%)	C	1	2.5	4

The total number of experiments was 15 based on three levels and a three-factor experimental design, with three replicates at the centre points. The enzyme activity analysis of each experiment was performed in triplicate and its average was obtained as the response (Y). Design Expert (version 10) was used to design the experiments and analyse data. The quadratic model for predicting the optimal point was express as follows:

$$Y = \beta 0 + \beta_1 A + \beta 2B + \beta 3C + \beta\ 12AB + \beta_{13}AC + \beta_{23}BC + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2$$

where Y is the response variables, A, B and C are coded level of independent variables, β_0 is the intercept term, β_1 β_2 and β_3 are linear coefficients, β_{11}, β_{22} and β_{33} are quadratic coefficients, β_{12}, β_{13} and β_{23} are interactive coefficient. Statistical analysis of the model was performed to analyse variance (ANOVA). F-value determines the statistical significance of the model, and the proportion of variance explained was achieved through the multiple coefficients of determination (R^2). The optimal values of these factors were determined by response surface and point prediction using Design Expert software. Three dimensional (3D) contour plots were used to represent the quadratic models of variables.

3.2.3.2 Incubation time

Selected cultures were incubated in 100 ml production medium in 500 ml Erlenmeyer flasks at 27^{o}C from day 0 to day 10 on a rotary shaker at 150 rpm. 5ml of the sample was withdrawn every day for testing enzyme activity by the DNS assay and cell density by UV Spectrometer at a wavelength of 600 nm.

3.3 Results and discussion

3.3.1 Isolation of actinobacteria from decomposing seaweed

Decomposing seaweed was plated onto three isolation media (GAA, SYP and HVA, and) in triplicate for each medium. Actinobacteria were grown during six weeks of incubation at 27^{o}C (Figure 3.1).

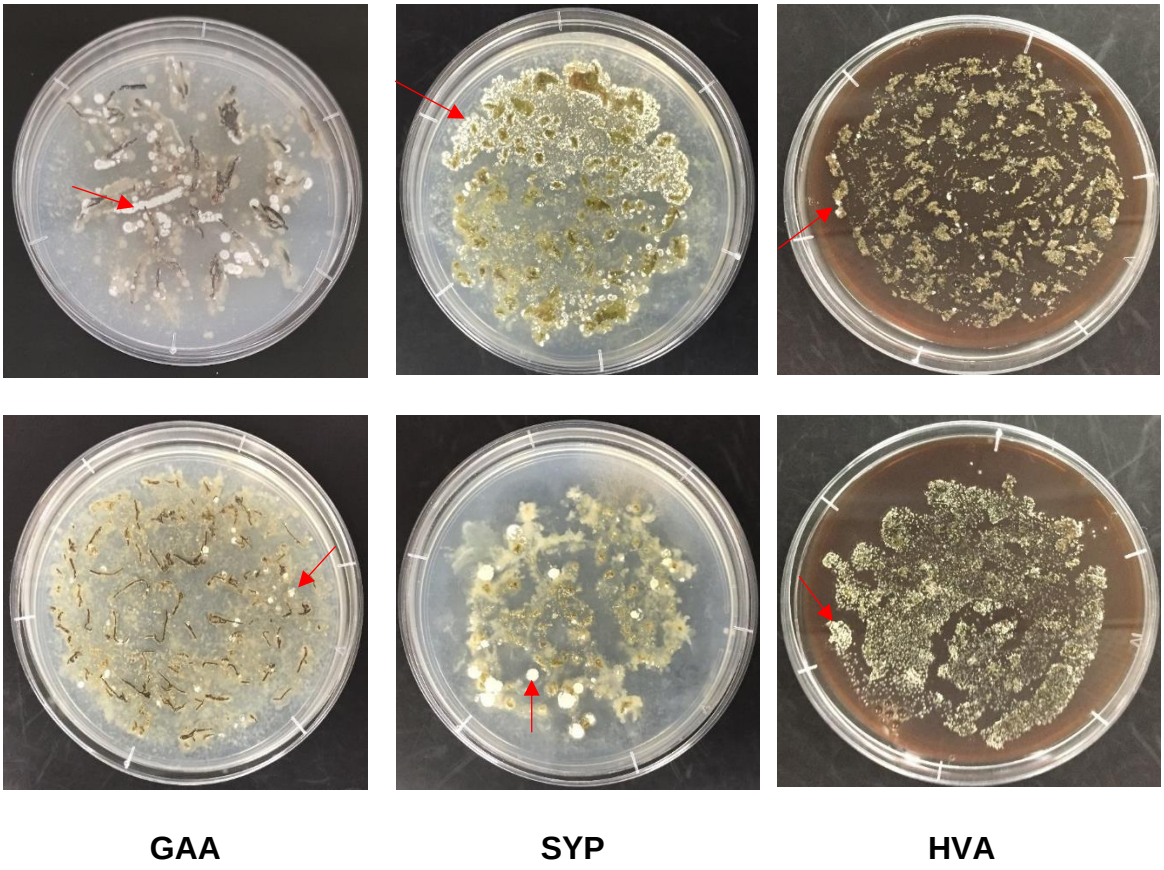


Figure 3.1: The growth of actinobacteria from decomposing seaweed on three different media.

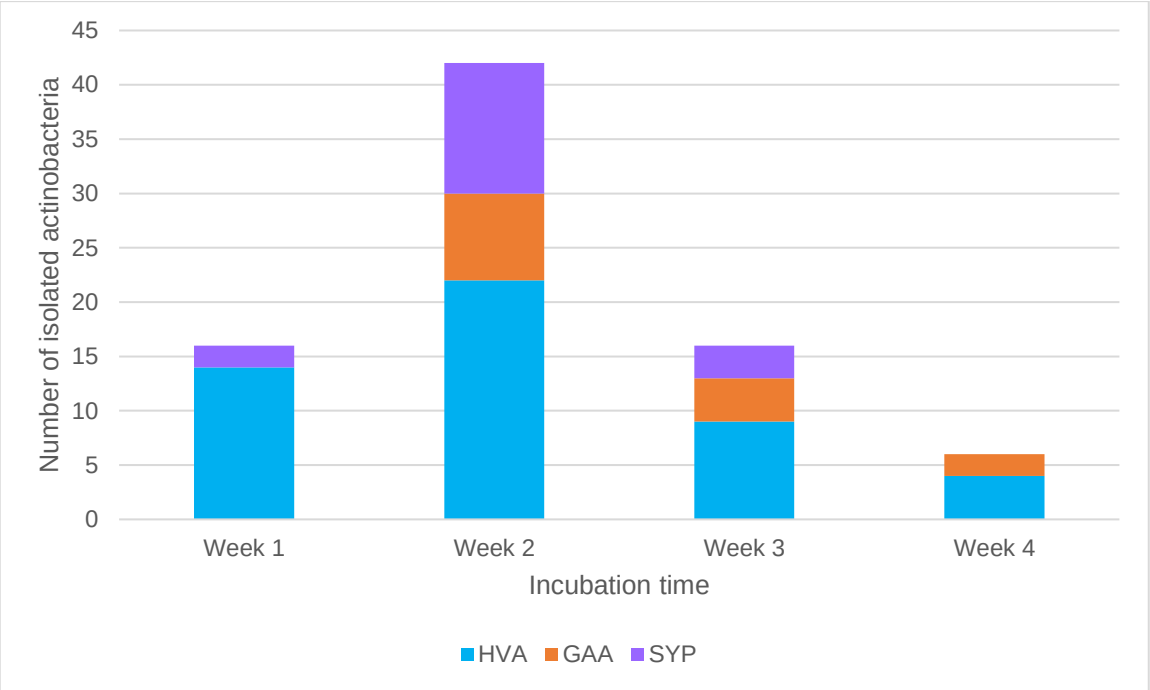


Figure 3.2: Number of actinobacterial strains isolated from decomposing seaweed on three different media (HVA, GAA and SYP) over four weeks.

All the colonies were picked from the three isolation media and purified by streaking onto half-strength potato dextrose agar (HPDA). There were eighty different strains isolated in which 49, 17, and 14 actinobacteria were isolated from HVA, SYP and GAA, respectively (Figure 3.2). The results indicated that HVA was the most effective isolation medium for actinobacteria that gave the highest number of isolates (61.3%) and less contamination compared with the other media (17.5% for GAA and 21.2% for SYP). These results are similar to the research of Alanazi (2013), Le *et al.* (2016) and Vo (2018) who isolated endophytic actinobacteria from South Australia seaweed, from legume plants and the roots of chickpea, respectively. Additionally, the highest number of isolates were obtained in the second week for all three isolation media. Some actinobacteria strains are displayed in Figure 3.3.

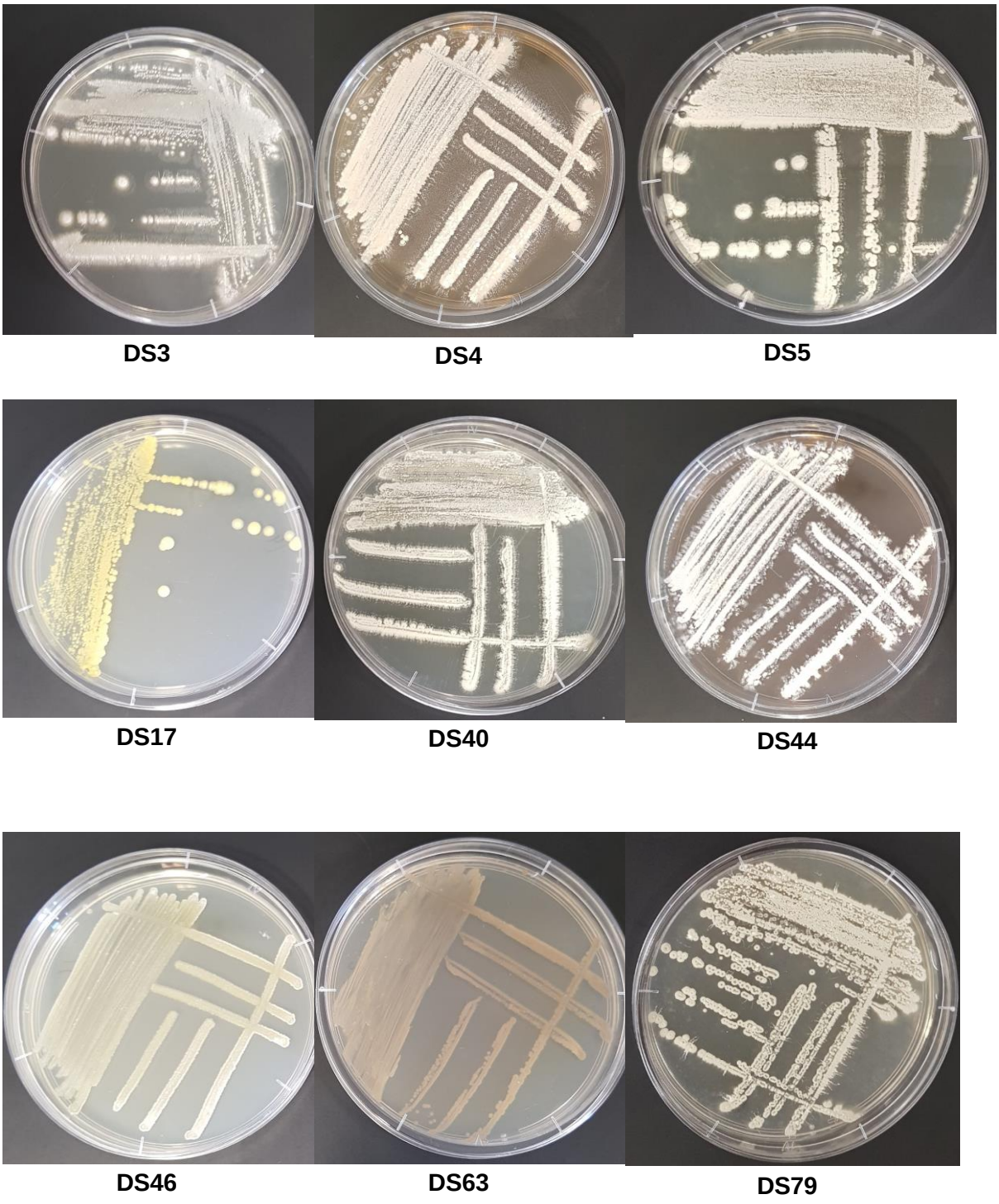


Figure 3.3: Actinobacterial strains isolated from decomposing seaweed on HPDA agar.

3.3.2 Screening for polysaccharide-degrading enzymes

Eighty strains of actinobacteria were inoculated in Bull Kelp medium at 27ºC with shaking at 250 rpm. Broth cultures after 7 days incubation were centrifuged at 10,000 g for 20 min at 4ºC. The cell-free supernatants after centrifugation considered as crude enzyme were collected and stored at 4ºC. The activities of polysaccharide-degrading enzymes including alginate lyase and fucoidanase extracted from eighty actinobacteria strains determined by DNS assay are shown in Table 3.2.

Table 3.2: The activity of Alginate lyase and fucoidanase of 80 isolated strains (n=3).

Strain number	Alginate lyase activity (U/ml)	Fucoidanase activity (U/ml)	Strain number	Alginate lyase activity (U/ml)	Fucoidanase activity (U/ml)
DS1	2.801 ± 0.27	1.107 ± 0.12	**41**	2.739 ± 0.26	1.041 ± 0.28
2	1.763 ± 0.33	1.064 ± 0.42	**42**	2.537 ± 0.17	1.524 ± 0.34
3	0.909 ± 0.21	2.328 ± 0.37	**43**	2.496 ± 0.23	2.257 ± 0.45
4	8.214 ± 0.16	2.833 ± 0.15	**44**	8.867 ± 0.12	3.216 ± 0.24
5	1.935 ± 0.25	1.535 ± 0.36	**45**	3.077 ± 0.15	0.304 ± 0.19
6	2.979 ± 0.19	2.429 ± 0.27	**46**	1.909 ± 0.39	0.112 ± 0.05
7	2.671 ± 0.42	1.394 ± 0.33	**47**	1.261 ± 0.16	2.082 ± 0.24
8	1.732 ± 0.27	1.118 ± 0.19	**48**	0.937 ± 0.17	1.829 ± 0.18
9	1.384 ± 0.30	1.073 ± 0.15	**49**	7.994 ± 0.22	2.401 ± 0.55
10	3.592 ± 0.46	2.195 ± 0.26	**50**	2.899 ± 0.45	0.817 ± 0.08
11	2.128 ± 0.18	1.758 ± 0.23	**51**	4.174 ± 0.33	2.015 ± 0.17
12	2.481 ± 0.12	1.526 ± 0.18	**52**	3.178 ± 0.26	2.345 ± 0.22

Strain number	Alginate lyase activity (U/ml)	Fucoidanase activity (U/ml)	Strain number	Alginate lyase activity (U/ml)	Fucoidanase activity (U/ml)
13	7.679 ± 0.22	2.911 ± 0.25	**53**	2.995 ± 0.48	2.153 ± 0.46
14	5.821 ± 0.51	1.603 ± 0.32	**54**	4.083 ± 0.52	1.700 ± 0.32
15	4.917 ± 0.36	1.027 ± 0.24	**55**	1.659 ± 0.21	0.841 ± 0.11
16	7.328 ± 0.19	2.644 ± 0.21	**56**	0.699 ± 0.18	2.220 ± 0.20
17	7.788 ± 0.17	2.412 ± 0.20	**57**	1.130 ± 0.12	2.240 ± 0.17
18	2.294 ± 0.23	0.952 ± 0.17	**58**	2.576 ± 0.24	0.817 ± 0.13
19	4.016 ± 0.31	1.615 ± 0.26	**59**	4.534 ± 0.36	2.967 ± 0.15
20	4.948 ± 0.14	3.078 ± 0.42	**60**	3.057 ± 0.24	2.096 ± 0.12
21	7.751 ± 0.17	2.719 ± 0.26	**61**	1.864 ± 0.17	0.691 ± 0.05
22	1.267 ± 0.17	2.816 ± 0.36	**62**	0.699 ± 0.12	0.817 ± 0.09
23	2.129 ± 0.24	1.032 ± 0.13	**63**	1.744 ± 0.25	1.414 ± 0.32
24	2.119 ± 0.12	1.359 ± 0.24	**64**	2.572 ± 0.16	1.237 ± 0.24
25	0.833 ± 0.13	1.001 ± 0.17	**65**	7.696 ± 0.23	3.028 ± 0.13
26	1.570 ± 0.15	1.202 ± 0.38	**66**	7.131 ± 0.45	2.981 ± 0.15
27	1.743 ± 0.37	1.094 ± 0.27	**67**	1.926 ± 0.21	1.653 ± 0.17
28	1.148 ± 0.24	1.494 ± 0.21	**68**	7.037 ± 0.11	2.436 ± 0.38
29	1.259 ± 0.39	0.792 ± 0.11	**69**	1.454 ± 0.23	1.043 ± 0.32
30	1.681 ± 0.41	2.224 ± 0.25	**70**	1.592 ± 0.25	0.901 ± 0.11
31	2.841 ± 0.45	1.374 ± 0.28	**71**	2.185 ± 0.31	1.457 ± 0.30
32	2.516 ± 0.32	1.028 ± 0.22	**72**	1.668 ± 0.14	1.046 ± 0.28
33	1.854 ± 0.28	0.845 ± 0.11	**73**	1.941 ± 0.34	1.209 ± 0.24
34	1.641 ± 0.27	0.796 ± 0.12	**74**	1.211 ± 0.18	1.202 ± 0.16
35	3.967 ± 0.28	1.273 ± 0.29	**75**	2.058 ± 0.29	1.883 ± 0.15

Strain number	Alginate lyase activity (U/ml)	Fucoidanase activity (U/ml)	Strain number	Alginate lyase activity (U/ml)	Fucoidanase activity (U/ml)
36	1.278 ± 0.14	1.839 ± 0.41	**76**	7.954 ± 0.26	3.151 ± 0.41
37	1.58 ± 0.16	1.161 ± 0.25	**77**	7.699 ± 0.17	2.017 ± 0.32
38	1.365 ± 0.31	1.284 ± 0.13	**78**	7.493 ± 0.24	2.202 ± 0.23
39	2.479 ± 0.35	1.350 ± 0.21	**79**	8.464 ± 0.29	3.416 ± 0.25
40	8.349 ± 0.29	3.672 ± 0.28	**80**	3.044 ± 0.42	1.339 ± 0.14

Overall, the results presented above show that all eighty strains have higher activity toward alginate lyase than fucoidanase. In which nineteen strains produced high alginate lyase activity (above 4 U/ml), accounting for 23.6% of the total. The highest activities noted were 8.349 U/ml, 8.867 U/ml and 8.464 U/ml that belonged to strains DS40, DS44 and DS79, respectively. Therefore, these three strains were chosen for the next step of alginate lyase purification. While enzyme activities toward alginate lyase were high among actinobacteria strains, they showed low activity in terms of fucoidanase. The highest activities of fucoidanase were also found from DS40, DS44 and DS79. These results suggested that DS40, DS44 and DS79 could be good producers of both alginate lyase and fucoidanase.

3.3.3 Optimisation of culture conditions

3.3.3.1 Composition of culture medium

Strains of DS40, DS44, and DS79 were marine actinobacteria isolated from degrading seaweed, so they were expected to have similar nutritional requirements. Therefore, strain DS40 was used for the optimisation of the composition of culture

medium. The Box Behnken design with three factors and three levels were used to determine the response (alginate lyase activity). Peptone (g/L), yeast extract (g/L), and NaCl (%) were independent variables, which studied the optimum concentration of these parameters. The predicted and experimental values of the variables for each experiment in the design matrix were obtained as shown in Table 3.3. ANOVA was performed to analyse the results from which the second-order polynomial equation was established for alginate lyase activity as follows:

$$Y\ (U/ml) = 6.20 - 0.60*A - 0.93*B - 1.17*C + 0.23*AB - 0.48*AC - 0.41*BC + 0.17*A^2 - 0.27*B^2 - 0.68*C^2$$

Where Y is the predicted alginate lyase activity, and A, B, C are peptone, yeast extract, NaCl, respectively. F-test and ANOVA from Design Expert 10.0 software were used to evaluate the statistical significance of the equation which was summarised in Table 3.4. The Model F-value for alginate lyase activity was 12.61 (p value < 0.05) implied that the model was significant. The coefficient (R^2) value was high (0.9578), explaining 95.78% of the variables peptone (A), yeast extract (B) and NaCl (C) were supported by the response (Y). This value indicated that the proposed experimental design was suitable for the simulation of alginate lyase activity (a value > 0.75 indicated aptness of the model (Pio & Macedo, 2008)).

Table 3.3: Experimental design and results of the Box-Behnken design for strain DS40 growing on 2% of Bulk Kelp production medium.

Run no.	Peptone (g/L)	Yeast extract (g/L)	NaCl (%)	Alginate lyase activity (U/ml)	
	A	B	C	Experimental	Predicted
1	15	5.5	3.5	3.02	3.44
2	10	5.5	2	5.77	6.20
3	5	5.5	3.5	5.57	5.59
4	5	10	2	5.40	5.53
5	10	5.5	2	6.57	6.20
6	5	1	2	7.59	7.86
7	5	5.5	0.5	7.41	6.99
8	15	10	2	5.08	4.80
9	10	1	3.5	5.71	5.41
10	10	10	3.5	2.88	2.73
11	15	1	2	6.33	6.20
12	15	5.5	0.5	6.76	6.74
13	10	10	0.5	5.61	5.90
14	10	5.5	2	6.26	6.20
15	10	1	0.5	6.79	6.79

The data in Table 3.3 showed that the maximum alginate lyase of DS40 could be achieved when the peptone, yeast extract and NaCl were set at 5 g/L, 1 g/L, and 2% (20 g/L), respectively.

Table 3.4: Analysis of variance (ANOVA) for predictive equation for the activity DS44 alginate lyase.

Factors	Coefficient	Standard error	F-value	p-value (P > F)
Intercept	6.20	0.2697	12.61	0.0061**
A-Peptone	-0.60	0.1652	13.14	0.0151*
B-Yeast extract	-0.93	0.1652	31.84	0.0024**
C-NaCl	-1.17	0.1652	50.58	0.0009**
AB	0.23	0.2336	1.00	0.3635
AC	-0.48	0.2336	4.15	0.0973
BC	-0.41	0.2336	3.12	0.1376
A^2	0.17	0.2431	0.50	0.5121
B^2	-0.27	0.2431	1.27	0.3108
C^2	-0.68	0.2431	7.88	0.0377*

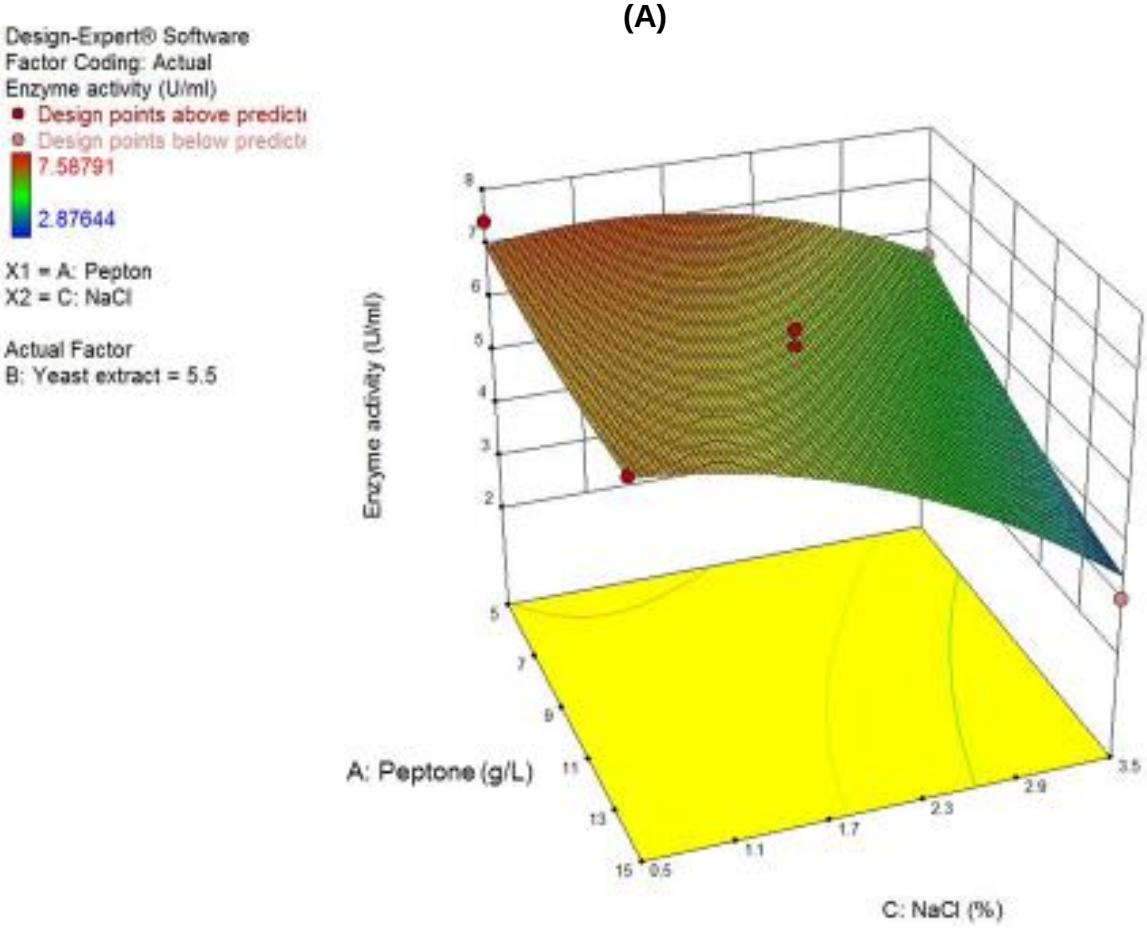
** P < 0.05, ** P< 0.01; Coefficient of determination* ***R^2 = 0.9578***

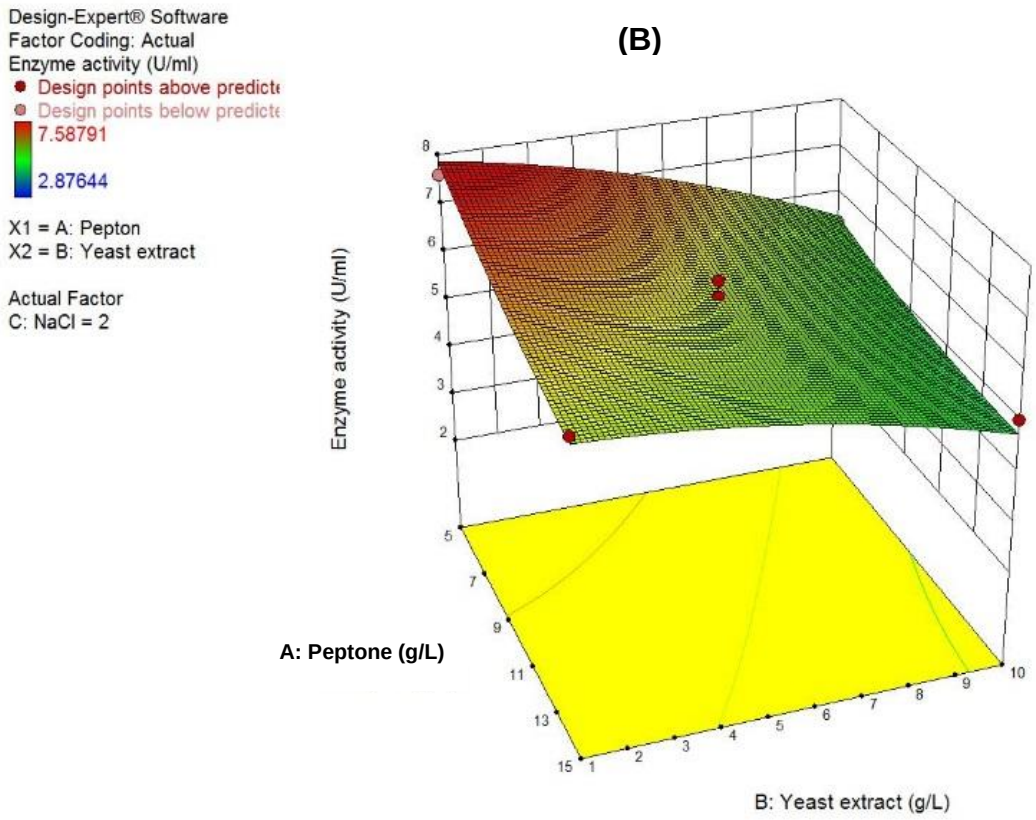
As has been proven, the smaller p value (p<0.05) indicated the high significance of the corresponding coefficient. In this research, all three variables studied had p-values less than 0.05, indicating significant model terms. As can be seen, the variables having the highest effect were NaCl (C) and yeast extract (B). However, no interactions exhibited p-values less than 0.05, showing that there was no statistically significant interaction. As the alginate lyase activity was the response (Y), the concentration of NaCl and yeast extract played an important role in its enhancement (p< 0.005).

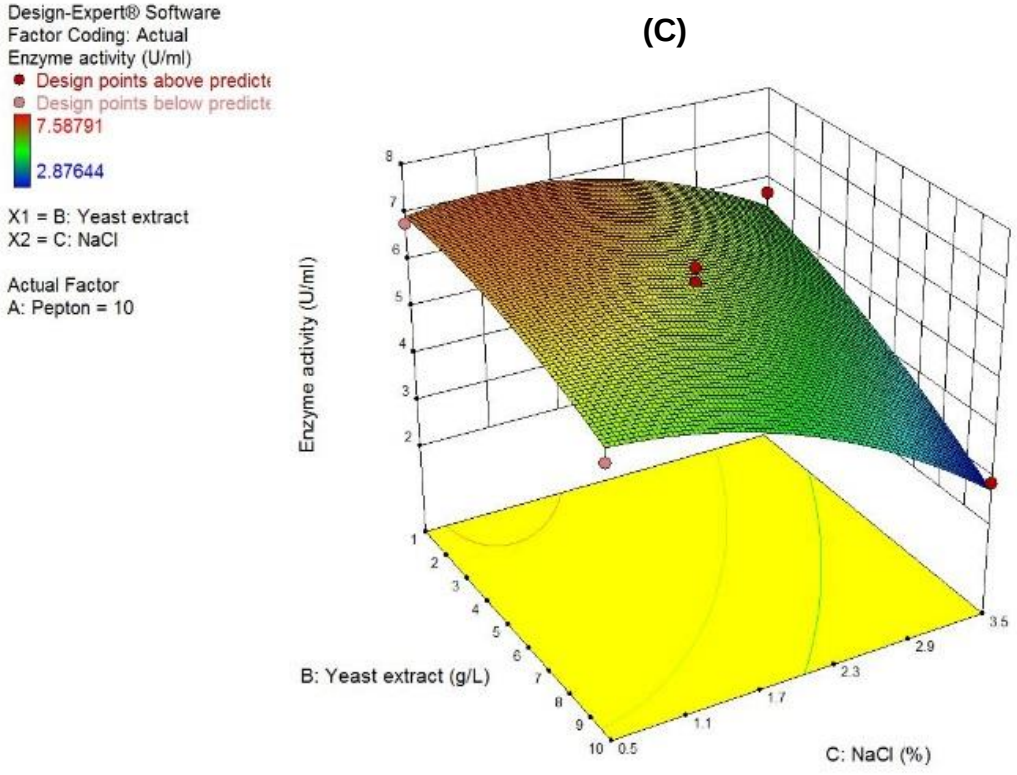
Three dimensional response surface plots and their respective contour plots graphically representing regression equations were created to demonstrate the interaction between two experimental variables and their effect on response (Singh *et al.*, 2015). The main goal of the response surface is to optimise the values of the variables to obtain an optimal response (Tanyildizi *et al.,* 2005). Each contour curve exhibits the effect of two factors, whereas the other factor will help at a zero level. Indicated maximum predicted value is by way of the surface confined within the smallest ellipse in the contour diagram. Perfect interaction between the independent variables must be present for Elliptical contours to be obtained (Muralidhar *et al.* 2001). Contour plot shown in Figure 3.4 A, B, & C were not elliptical in shape because they did not demonstrate a statistically significant association between peptone and NaCl, yeast extract and NaCl, peptone and yeast extract. Figure 3.4 A & B also had a similar pattern, when peptone and yeast extract set to the minimum concentration of 5 g/L and 1 g/L, respectively, an increase in the concentration of NaCl led to an increase in alginate lyase activity to a limited degree, by further increasing its concentration, this led to a decrease in enzyme activity. For an explanation in the results, strain DS40 is a marine actinobacterium which isolated from degrading seaweed that gave the best growth and produced enzyme at a high yield at the appropriate concentration of NaCl. Any further addition of NaCl could cause the inhibition of the enzyme production. As shown in Figure 3.4 C, the maximum activity was obtained when peptone and yeast extract were set at the minimum concentration. Therefore, there was a decrease in the enzyme activity with increases in the concentration of peptone and yeast extract. Figure 3.4 D showed the correlation between the predicted and experimental response for alginate lyase

activity. Distribution of values around the regression line represented the relationship between predicted and experimental responses.

(A)




Design-Expert® Software
Factor Coding: Actual
Enzyme activity (U/ml)
Design points above predicte
Design points below predicte
7.58791
2.87644
X1 = A: Pepton
X2 = B: Yeast extract
Actual Factor
C: NaCl = 2
(B)
Enzyme activity (U/ml)
A: Peptone (g/L)
B: Yeast extract (g/L)


Design-Expert® Software
Factor Coding: Actual
Enzyme activity (U/ml)
Design points above predicte
Design points below predicte
7.58791
2.87644
X1 = B: Yeast extract
X2 = C: NaCl
Actual Factor
A: Pepton = 10
(C)
Enzyme activity (U/ml)
B: Yeast extract (g/L)
C: NaCl (%)

(D)

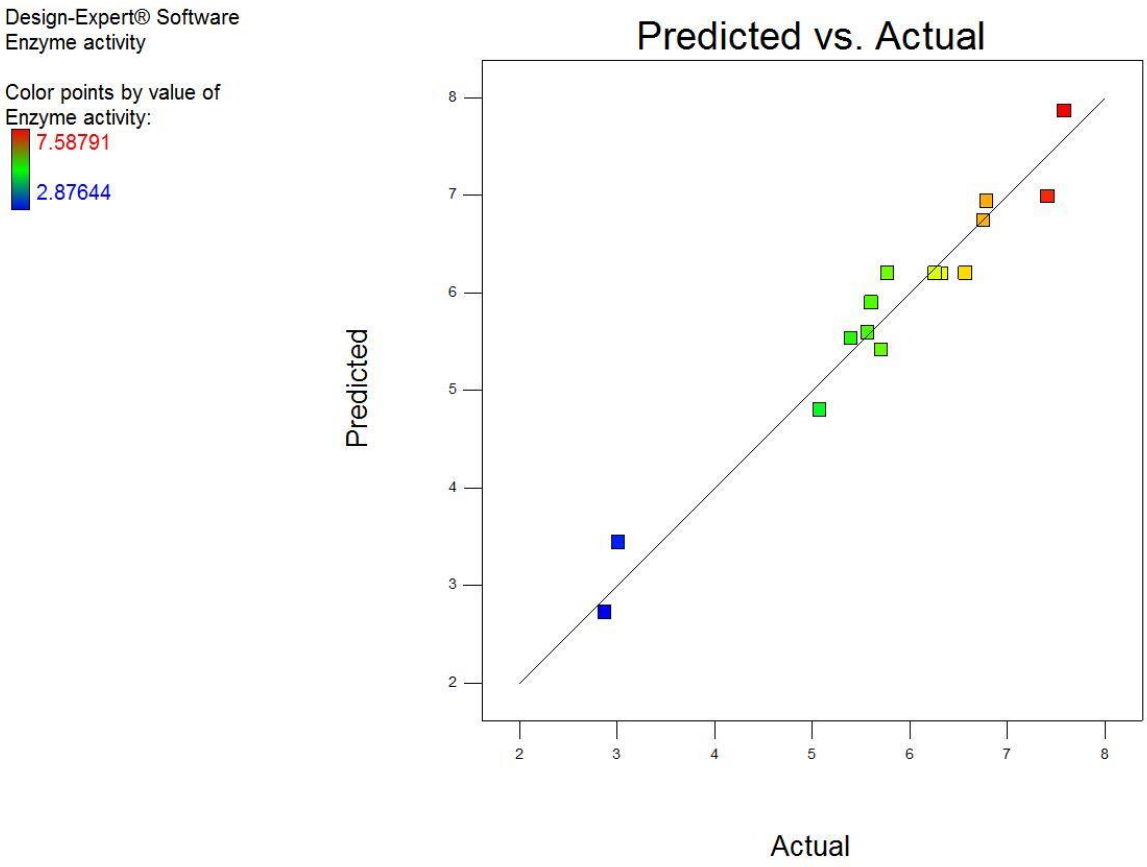
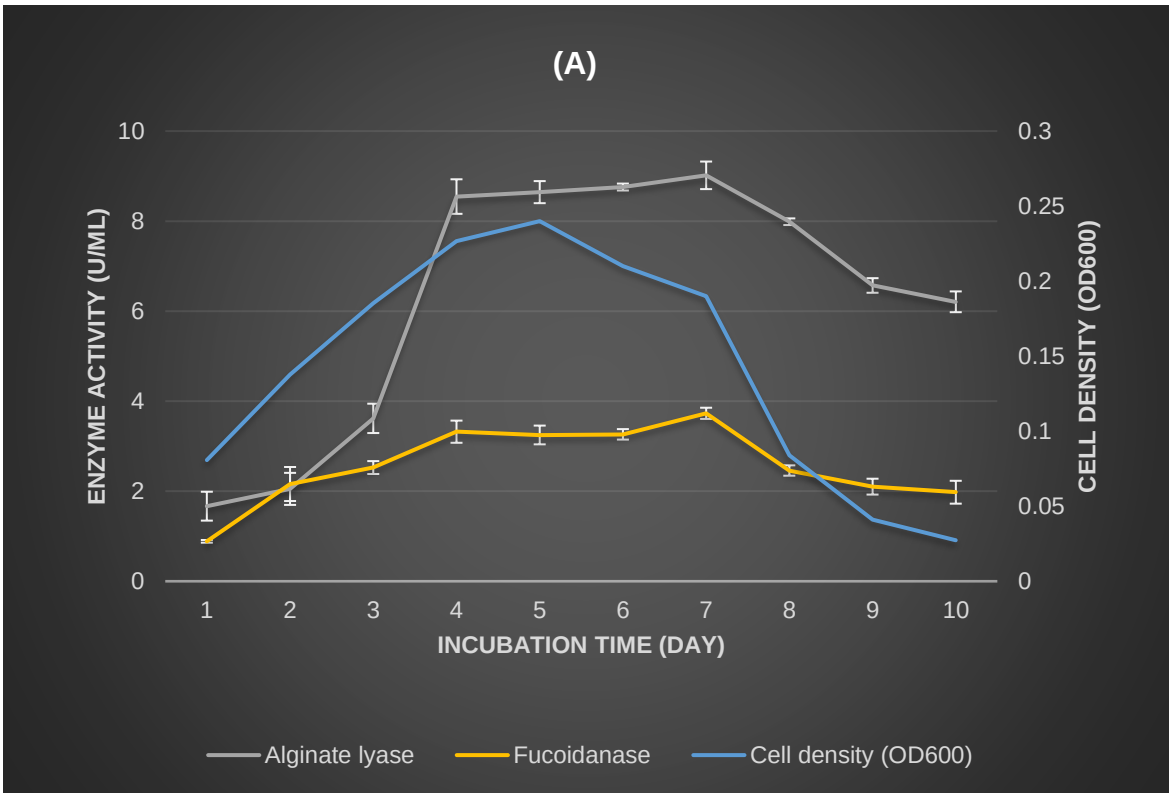
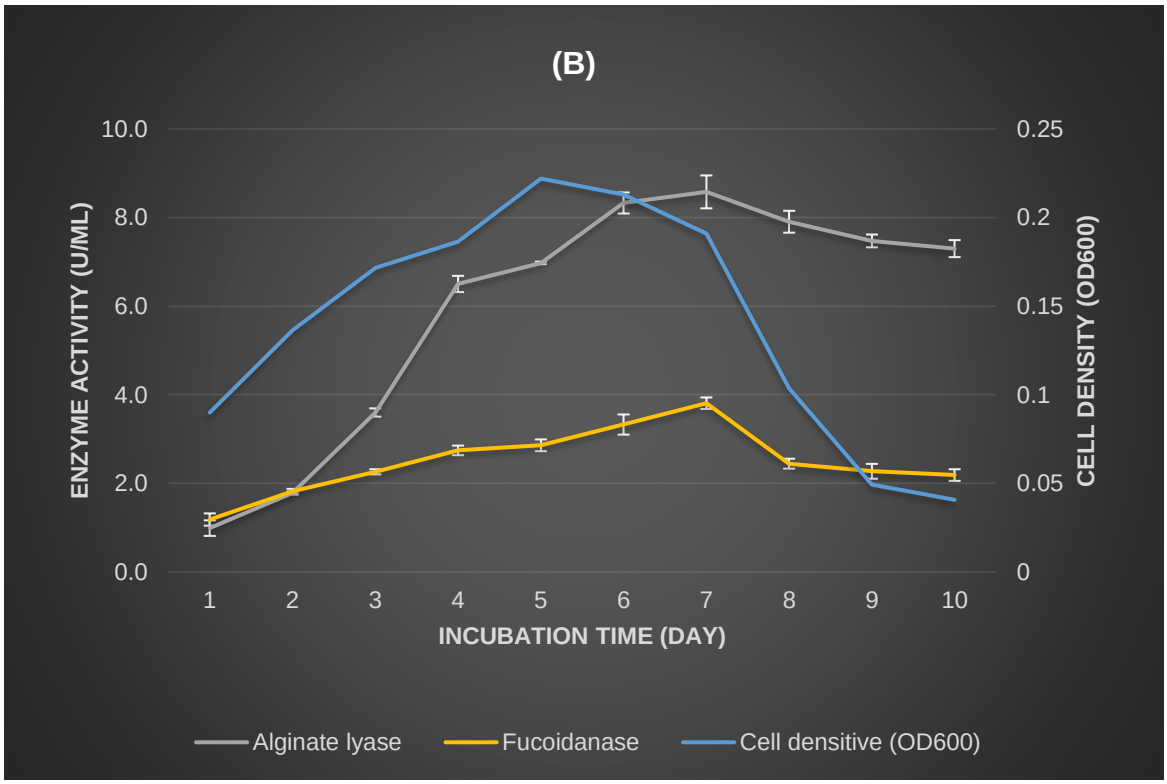


Figure 3.4: Response surface 3D plot showing the interaction of peptone, Yeast extract and NaCl on the enzyme activity (A, B, C). Relationship between predicted and experimental response for enzyme activity (D).

3.3.3.2 Incubation time

Three strains DS40, DS44, and DS79 were inoculated into 50 ml inoculum medium IM22 and cultured at 27^{o}C with shaking at 150 rpm. After three days, 5 ml of the inoculum were transferred to 100 ml production medium in 500 ml Erlenmeyer flasks and then incubated at 27^{o}C from day 0 to day 10 on a rotary shaker at 150 rpm. The enzyme activity of daily samples analysed by DNS assay and their cell density measured by UV Spectrometer at wavelength of 600 nm are exhibited in Figure 3.5.


(A)
ENZYME ACTIVITY (U/ML)
CELL DENSITY (OD600)
INCUBATION TIME (DAY)
Alginate lyase
Fucoidanase
Cell density (OD600)


(B)
ENZYME ACTIVITY (U/ML)
CELL DENSITY (OD600)
INCUBATION TIME (DAY)
Alginate lyase
Fucoidanase
Cell densitive (OD600)

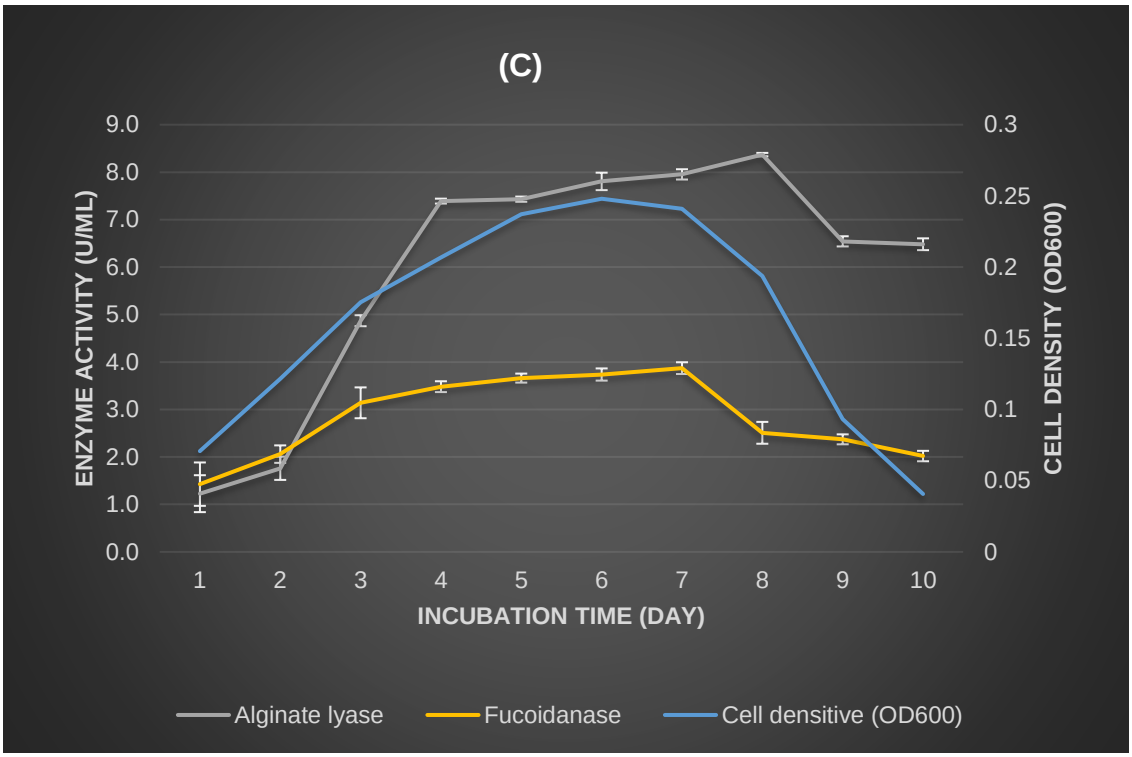


Figure 3.5: Enzyme activity of alginate lyase and fucoidanase, and cell density of strains DS40 (A), DS44 (B) and DS79 (C) from day 1 to day 10 of incubation at 27^{0}C with shaking at 150rpm. Error bar show standard error of mean, n=3.

It can be seen in Figure 3.5, enzyme activity toward alginate lyase increased dramatically after four days of incubation and reach to a peak at 7th day for two strains DS40 and DS44, accounting for 9.017 U ml^{-1} and 8.079 U ml^{-1}, respectively. Alginate lyase of DS79 can be extracted with the highest activity (8.369 U ml^{-1}) after eight days at 27^{o}C. In terms of fucoidanase, the enzyme activity of all three strains increased slightly and had a maximum oh the 7th day of incubation. Additionally, these graphs also showed that enzyme synthesis closely paralleled the growth phase. The enzymes were synthesised during the exponential phase leading to the improvement of enzyme activity at this stage. While the cells entered the stationary phase of growth, the extracellular enzyme activity reached a maximum. Therefore, it can be concluded that enzyme synthesis increases at the same time as the growth of actinobacteria.

3.4 Conclusion

In conclusion, seaweed was found to be an excellent source to yield actinobacteria which could be a potential resource for the discovery of novel polysaccharide-degrading enzymes. Eighty strains of actinobacteria isolated from decomposing seaweed have been obtained in which strains DS40, DS44, and DS79 exhibited highest activity in terms of alginate degradation. The optimum culture conditions for these strains contained the main ingredients of 5 g/L peptone, 1g/L yeast extract and 2% NaCl. They could produce the highest enzyme activity after seven days incubation at 27^{o}C toward fucoidanase for all three strains. The alginate lyases showing the highest activity could be obtained by strains DS40 and DS44 after seven days and by strain DS79 after eight days.

CHAPTER 4: PURIFICATION AND CHARACTERISATION OF ALGINATE LYASE FOR HYDROLYSING ALGINATE

4.1 Introduction

The primary aim of the purification process is to recover the target protein(s) from thousands of proteins present in the fermentation broth. The target enzymes have the advantages of being detected due to their high catalytic activity and specificity.

There are various techniques that are used to separate the protein depending on their chemical and physical properties. The first step is the precipitation of the protein by salting them out using ammonium sulphate, which is the most common method (Bodzon-Kulakowska *et al.*, 2007). After precipitation with ammonium sulphate, the protein is dialysed to remove salts associated with the protein. Among the newer technology methods for further purification of protein, Fast Protein Liquid Chromatography (FPLC) is widely used for carrying out ion exchange chromatography, size exclusion chromatography, hydrophobic interaction chromatography and affinity chromatography. These methods separate proteins according to their charge, size or binding affinity.

Depending on the source of alginate lyase, there are many different characteristics of the enzymes in terms of the mode of action and specific substrates. The alginate lyases can have superior degradation activity against either polyM or PolyG-block. However, some alginates lyases can degrade both polyM and polyG-block (polyMG-specific lyase) that show bifunctional activity. On the other hand, alginate lyase can be classified as an exo- or endo-acting enzymes. In recent times, the exo-type lyases have rarely been reported and characterized (Kim *et al.*, 2012; Ochiai *et al.*, 2010; Park *et al.*, 2012). Therefore, finding new alginate lyases and their characterisation would provide the

association between the biological activities and the structures of alginate. This can bring significant advantages for both academic researchers and industrial companies. Additionally, the analysis of protein components requires spectroscopic methods, with mass spectrometry (MS) being the most powerful and effective (Schneider, 2010).

This chapter focuses on purification and characterisation of alginate lyase from three different strains of actinobacteria that showed the highest activity in hydrolysing alginate.

4.2 Materials and Methods

4.2.1 Purification of enzymes.

The crude enzymes were precipitated by ammonium sulphate at concentrations of 20%, 30%, 40%, 50%, and 60% and precipitates dialyzed using cellular membrane (14,000 molecular weight cut off). This partially purified enzymes were further purified by anion-exchange chromatography (Mono Q^{TM} 5/50 GL column) and size exclusion chromatography (Superdex^{TM} 75 10/300 GL column). All these purification methods were explained in section 2.5.

4.2.2 Characterisation of purified enzymes.

4.2.2.1 Molecular weight

Enzyme molecular weight was carried out by SDS-PAGE. This methods were explained in section 2.6.1.

4.2.2.2 Amino acid sequences

The amino acid sequences of polysaccharide-degrading enzymes were identified by using 5600+ qTOF mass spectrometry (AB Sciex, Framingham, MA, USA) as per the following method:

- After running 1D SDS-PAGE, the gel was stained with Coomassie Brilliant Blue R-250. The gel was placed into Coomassie blue stain solution (Appendix D4) for 30 minutes with shaking. It was transferred into dH_2O and placed into microwave for 20 minutes. The pre-warmed gel was subsequently transferred into destain solution (20% methanol and 7.5% acetic acid).

- For preparing samples to run mass spectrometry (MS), the gel bands of interest were cut out and put into a 1.5ml tube. The gel bands were processed by the following steps:

- **Washing**: Gel pieces were washed with 200µl of water with mixing by vortex for 1 minute. After washing, the water discarded and the gel destained with 100µl of acetonitrile (hypergrade, ACN):50mM ammonium bicarbonate (ABC) (1:1 v/v) for 2 minute with mixing by vortex. The supernatants were discarded. This step was repeated twice. The gel pieces were dehydrated for 5 minutes in 100µl of ACN:50mM ABC (1:1 v/v) with mixing by vortex. The supernatants were discarded. 100µl of 100% ACN was added, samples were mixed and then incubated for 1 minute. The supernatants were discarded.

- **Reduction/Alkylation:** The gel pieces were rehydrated in 100µl of freshly prepared 50mM DTT in 50mM ABC, and incubated for 20 minutes at 56°C. The supernatants were discarded, and 100µl of freshly prepared 100mM iodoacetamide in 50mM ABC was added, then samples were incubated in the

dark for 20 minutes at room temperature. The supernatants were discarded and the gel pieces were washed with 400µl of water with mixing briefly by vortex. The supernatants were discarded, and the washing step was repeated once. The gel pieces were dehydrated for 5 minutes in 100µl of ACN:50mM ABC (1:1 v/v) with intermittent vortex mixing. The supernatants were discarded. 100µl of 100% ACN was added, the samples were mixed and incubated for 1 minute. The supernatants were discarded, and the samples were dried in a 37°C oven for 5 minutes.

- **Digestion:** Trypsin (Promega) working solution was prepared by diluting 1 µL of stock solution (1 µg/µL in 2 µL aliquots) in 77.4 µL of 50mM ABC and 1.6 µL of 50mM $CaCl_2$ to produce a 12.5ng/µL working solution. The gel pieces were rehydrated with enough 12.5 ng/µL trypsin to rehydrate the gel pieces for 10 minutes. It was overlaid with 20µl of 50mM ABC and gently mixed for several seconds. The samples were incubated at 37°C overnight. The samples were centrifuged at 6000 rpm for 10 seconds. After that, the extracted peptides in the digestion reaction were transferred into a new tube and centrifuged at maximum speed for 10 minutes. The supernatant was removed and placed into mass spec vials by pipetting from above the bottom of the tube, being careful to not disturb any pieces of acrylamide that may be present at the bottom of the tube.

- **Injection:** The samples were loaded into 5600+ qTOF mass spectrometry (AB Sciex, Framingham, MA, USA) fitted with an Ekspert nano LC 415 high performance liquid chromatography (HPLC) (Eksigent, AB Sciex). The samples were injected to a C18 trap (Eksigent, USA) and then eluted with a gradient of 2 – 40% acetonitrile gradient in a 15 cm x 75 µm C18 column (Nikkyos

Technos, Japan). By operating the instrument by use of high sensitivity positive ion mode with charge state of +2 to +5 ions selected, with one MS scan followed by 20 MS/MS scans.

- The results were analysed by Protein Pilot software (version 4.5). First of all, amino acid sequences of actinobacteria were downloaded from the Uniprot database of protein sequences. With these protein databases and experimental spectra, the search engine evaluates the similarity between the experimental spectrum and the (many) theoretical spectra and then identifies the best matching peptide for each experimental spectrum (Figure 4.1).

Figure 4.1: An illustration of peptide-spectrum matching. This figure has been reproduced from Granholm (2014).

4.2.2.3 Gene sequences

Amino acid sequences (12-26 amino acid) were chosen from mass spectrometry results, and DNA sequence were converted using codon usage table for the high % G+C *Streptomyces* (Wright & Bibb, 1992). The signal alginate lyase gene was found by comparing the longest DNA sequence (18-22 nucleotides) with the whole genome using the Geneious software.

After getting the full alginate lyase gene sequence, the full amino acid sequence was found by using translation tool ExPASy (Expert Protein Analysis System) to convert DNA to protein.

4.2.2.4 Multiple sequence alignment

The identified sequences were analysed to expose the similar sequences to reported alginate lyases. Amino acid sequence of alginate lyase AlyVI from *Vibrio* sp. QY101 (Genbank: AAP45155.1), AlyVOA from *Vibrio* sp. O2 (Genbank: ABB36771.1), A9mT from *Vibrio* sp. A9m (Genbank: BAH79132.1), AlgNJU-03 from *Vibrio* sp. NJU-03 (ASA33933.1) and ALG5 from *Streptomyces* sp. ALG5 (Genbank: ABS59291.1) were used for multiple sequence alignment with the project alginate lyases.

4.2.2.5 Primers encode alginate lyase gene

Primers to amplify alginate lyase gene were designed following the comparison of sequences from multiple strains by using Geneious Prime software.

4.2.2.6 Substrate specificity of alginate lyase

To identify substrate specific of alginate lyase, three different substrates including sodium alginate, polymannuronic (PolyM) and polyguluronic (PolyG) were used. Each reaction contained 100 µl each purified enzyme and 500 µl 0.5% each substrate in 0.02 M Tris-HCl buffer (pH 8.0). It was incubated at 37^{o}C for 2 hours and then enzyme activity was performed by DNS assay as described in section 2.3.2.

4.2.2.7 HPLC analysis of alginate lyase degradation products

To identify alginate lyase activity, molecular weight of degradation products were determined by HPLC (Shimazu, USA) with a GPC/ SEC column: PL aquagel-OH Mixed-H 8 um 300 $\times$ 7.5 mm (6 kDa - 10 MDa) and PL aquagel-OH 30 8 um 300 $\times$

7.5 mm (0.1 - 60 kDa). The mobile phase was 0.1 M sodium nitrate (Isocratic) which was filtered through a 0.45 um filter using clean glassware (HPLC-only vacuum filtration set) and de-gassed immediately prior to use. The standards and tested samples were dissolved in mobile phase to avoid the interference of negative peak from water. For preparing the sample, 100 µl of purified enzyme was incubated with 500 µl of different substrates including 0.5% sodium alginate, 0.5% PolyM and 0.5% PolyG in 0.02 M Tris-HCl buffer (pH 8.0) at 37^{o}C. 100 µl of sample were taken at 0h and 24h incubation and diluted two time with 0.1 M sodium nitrate. The sample was centrifuged at maximum speed (12,000 rpm) for 5 minutes and then 100 µl of sample was placed into HPLC vial (ensuring there are no suspended solids, and no air bubbles at the bottom of the vial). 50 µl of sample was injected and run through the column with a flow rate of 1.0 ml/min. The peaks were detected by refractive index (RI) detector.

4.2.2.8 ESI-MS analysis of alginate lyase degradation products

Electrospray-ionization mass spectroscopy (ESI-MS; Waters Synapt HDMS) were used to analyze the mass/charge ratio of the alginate lyase degradation products. Samples were run in negative ionization modes. A reaction containing 0.5% sodium alginate in 0.02M Tris-HCl buffer (pH 8.0) was incubated with purified enzyme at 37^{o}C for 24 hours. Samples were diluted in 1:50 methanol and then injected into electrospray. Mass calibration was performed via injection of sodium chloride. Mass range of the scans was from m/z 150 to 2000.

4.3 Results and discussion

4.3.1 Precipitation by ammonium sulphate

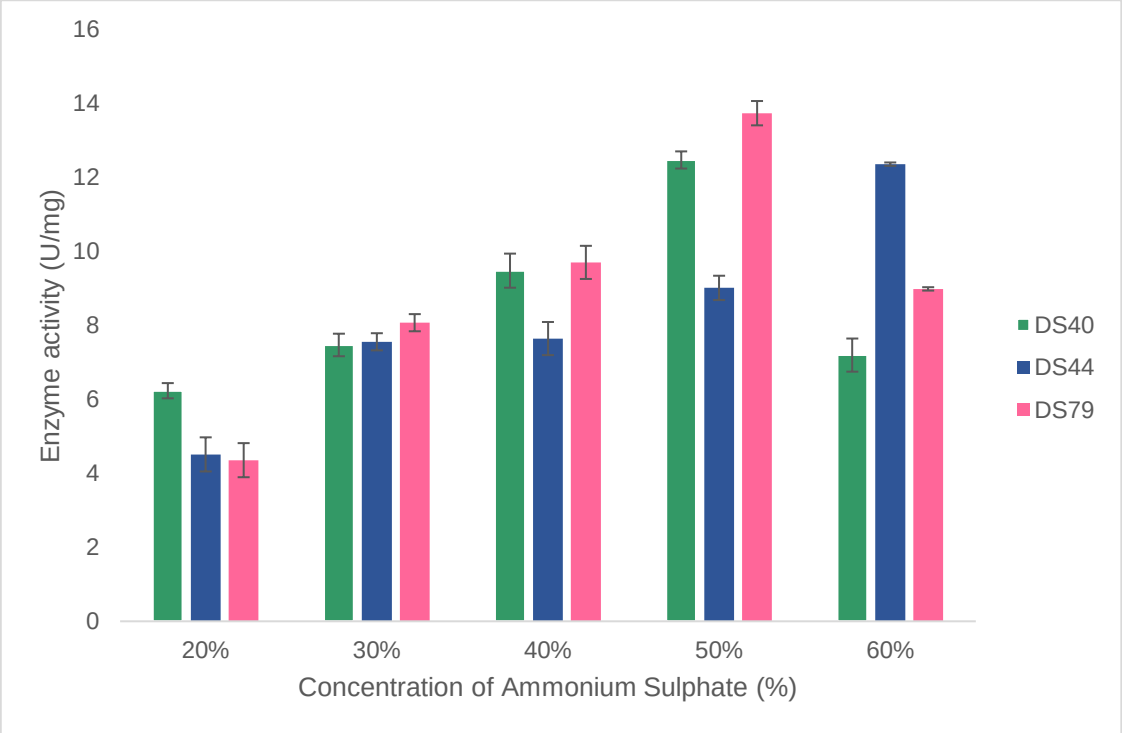


Figure 4.2: The enzyme activity of alginate lyase extracted from strains DS40, DS44 and DS79 and precipitated with five different concentrations of ammonium sulphate (22%, 30%, 40%, 50% and 60%). Error bars showed standard error of mean, n=3.

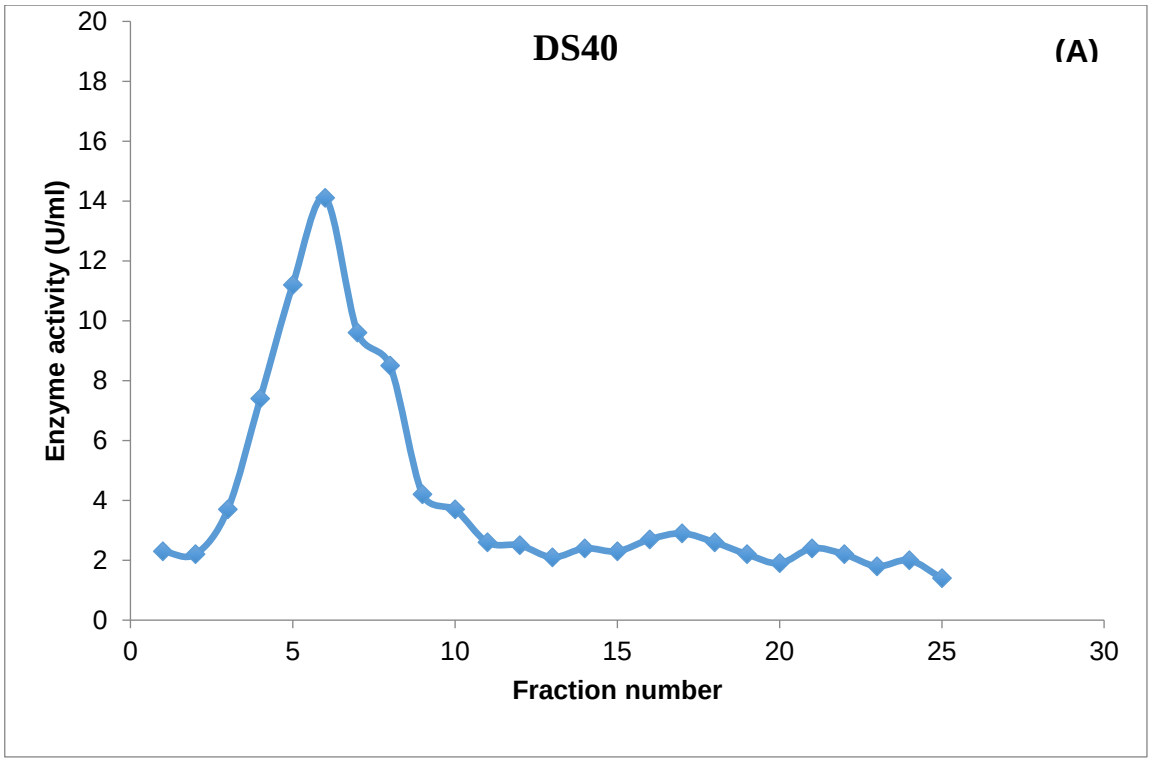
To optimize the ammonium sulphate concentration, the alginate degrading enzymes were precipitated from the culture filtrate of strains of DS40, DS44 and DS79 at five concentrations of ammonium sulphate (20%, 30%, 40%, 50% and 60%) and enzyme activities were assessed after dialysis (Figure 4.2). Alginate lyase from strains DS40 and DS79 grown on 2% Bull Kelp powder showed the highest activity when precipitated in 50% ammonium sulphate fraction (after dialysis), accounting for 12.474 U/ml and 13.739 U/ml respectively. Besides that, 60% ammonium sulphate was the optimum concentration to get partially purified alginate lyase from strain DS44. These results are similar with the study of Dou (2013), which the crude

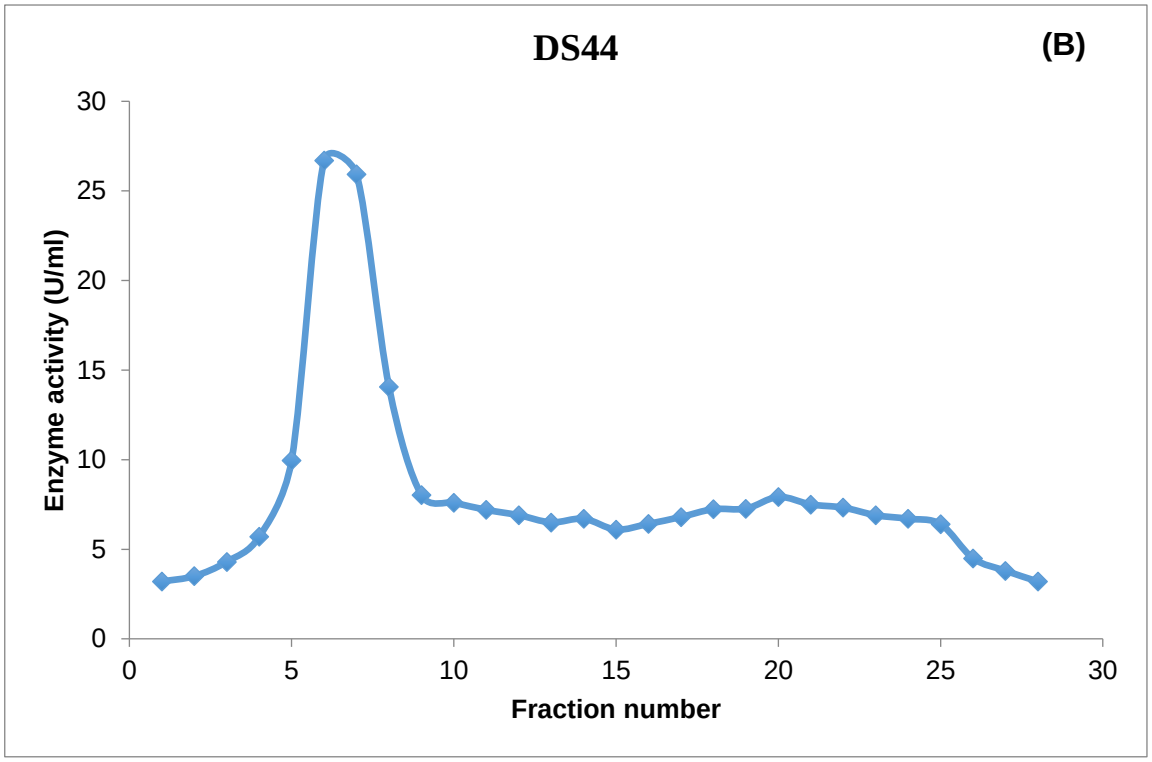
enzyme of alginate lyase from *Isoptericola halotolerans* CGMCC 5336 was precipitated by adding ammonium sulphate to obtain 50% to 70% saturation. The other references also showed that the alginate lyase was generally precipitated at high ammonium sulphate concentration of 75% such as the enzymes from marine *Microbulbifer* sp. ALW1 and *Vibrio furnissii* H1 (Zhu *et al.*, 2018; Zhu *et al.*, 2016).

Based on ANOVA statistical analysis, the activity of the enzymes precipitated by various concentration of ammonium sulphate were significantly different from each other except alginate lyase from strain DS44 when it was precipitated in 30% and 40% ammonium sulphate.

4.3.2 Purification of enzymes

For further purification of alginate lyases, the partially purified enzymes were first subjected to anion exchange column which was previously equilibrated with buffer A (20 mM Tris-HCl, pH 8). The fractions (corresponding to one major peak; as shown in a figure 4.3 A) were obtained using a NaCl linear gradient (0.2-0.3 M) from the column and fractions were assessed for enzyme activity. For strains DS40 and DS44, the highest activities were from fraction 6 at 45.75 U/mg and 93.78 U/mg respectively (Figure 4.3 A, B and Table 4.1 A, B). Fraction 8 which was collected from strain DS79 contained the highest activity, reaching 76.35 U/mg (Figure 4.3 C and Table 4.1 C). However, when on SDS PAGE, there were some other protein bands beside the target one. The fractions from peak were pooled and after being desalted, further purification of those fractions was achieved by size exclusion column (Superdex 75). The results of the purification of the alginate lyase are summarised in Table 4.1, indicating approximately 115 – 230 mg of proteins were purified from 1L of culture broth.


DS40
(A)
Enzyme activity (U/ml)
Fraction number
0
2
4
6
8
10
12
14
16
18
20
0
5
10
15
20
25
30


DS44
(B)
Enzyme activity (U/ml)
Fraction number
0
5
10
15
20
25
30
0
5
10
15
20
25
30

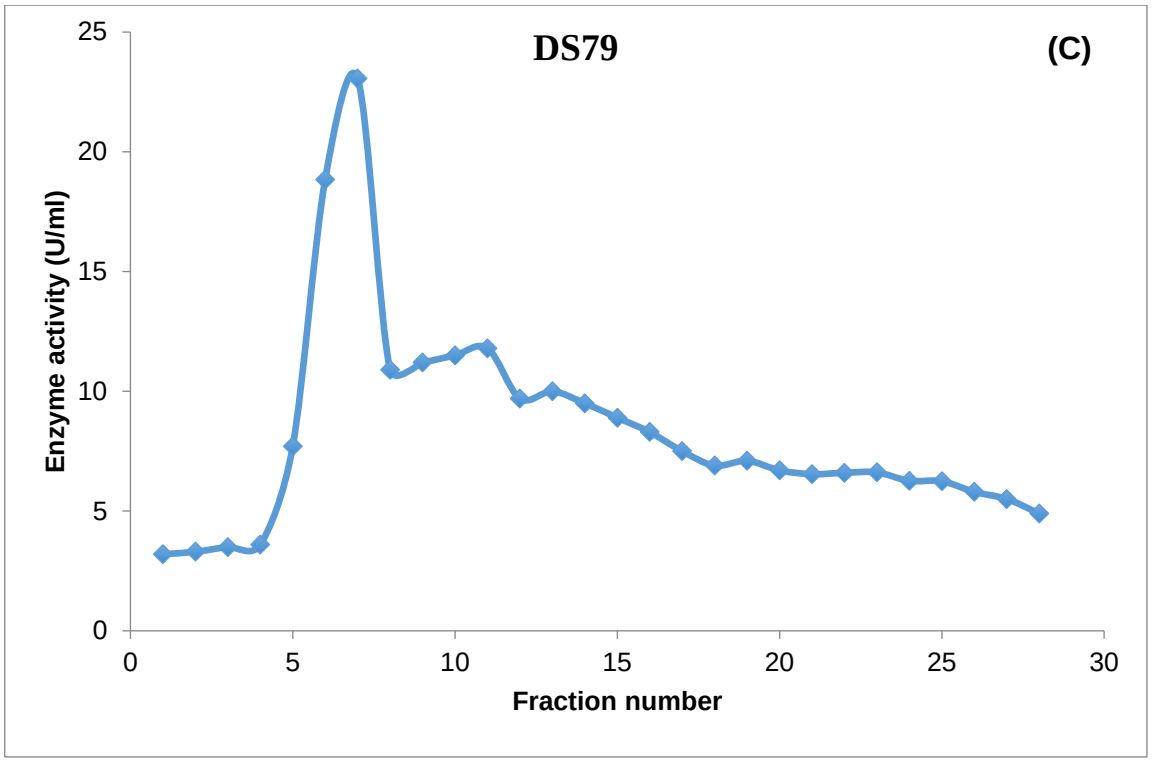


Figure 4.3: The enzyme activity of eluted fractions. The activity of purified enzymes from strains DS40 (A), DS44 (B) and DS79 (C) using anion exchange chromatography on a Mono-Q column.

Table 4.1: Summary of purification of alginate lyase from strains DS40 (A), DS44 (B) and DS79(C) (n=3).

(A)

Purification Steps	V (ml)	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (Fold)
Crude [a]	300	539.7	1457	2.70	100	1
50% Ammonium sulphate [b] (after dialysis)	12	11.00	269.7	24.5	18.51	5.85
Mono-Q [c]	1	0.305	13.96	45.75	0.96	16.94
Superdex 75 [d]	1	0.125	8.145	67.75	0.56	24.64

(B)

Purification Steps	V (ml)	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (Fold)
Crude [a]	300	604.5	695.9	1.01	100	1
60% Ammonium sulphate [b] (after dialysis)	12	13.52	370.8	27.54	53.3	27.27
Mono-Q [c]	1	0.327	28.88	93.78	4.15	92.85
Superdex 75 [d]	1	0.115	12.49	108.6	1.79	107.2

(C)

Purification Steps	V (ml)	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (Fold)
Crude [a]	300	601.5	1260	2.09	100	1
50% Ammonium sulphate [b] **(after dialysis)**	12	14.292	255.5	17.88	20.28	8.55
Mono-Q [c]	1	0.302	23.06	76.35	1.83	36.53
Superdex 75 [d]	1	0.231	23.97	103.4	1.90	49.47

[a] Crude enzyme from the culture broth

[b] Partial pure enzyme after ammonium sulphate precipitation and dialysis

[c] Active fraction obtained by anion exchange chromatography using Mono-Q column

[d] Active fraction obtained by size exclusion chromatography using Superdex 75 10/300 GL column

The alginate degrading enzymes were successfully purified from the culture broth of strains of DS40, DS44, and DS79 alginate lyases as shown in Table 4.1. The specific activity after purifying by anion exchange chromatography and size exclusion chromatography were very much higher than that of crude enzymes and dialysed enzymes. Alginate lyase from the three strains had relatively low yield due to the loss of protein when they were purified by chromatography techniques, however, their purity was high. Alginate lyase from strain DS44 exhibited the highest activity after each step, in comparison with the other two strains. It was purified 92.85-fold and 107.2-fold at a yield of 4.15% and 1.79%, and the specific activity of 93.76 U/mg and 108.6 U/mg by anion exchange chromatography and size exclusion chromatography, respectively.

Anion exchange chromatography is shown as an effective method for protein purification relating to their charge (Aguilar *et al.*, 2006). In a previous study, Rahman *et al.* (2010) had used Mono-Q column chromatography to purify alginate lyase from the common sea hare *Aplysia kurodai*. The target proteins were eluted with a linear gradient of 0 – 0.2 M NaCl at a flow rate of 15 ml/hour in 10 mM sodium phosphate buffer (pH 8.0). Two peak fractions were detected separately at 0.05 and 0.10 M NaCl of elution showed alginate lyase activity. In this study, the enzymes were eluted with a linear gradient of 0 – 1 M of NaCl in 50 mM Tris-HCl buffer (pH 8) at a flow rate of 0.5 ml/min. The highest enzyme activities of three strains were all detected in the first peak that eluted out at between 0.05 and 0.35 M (Appendix C1). This demonstrates that the proteins bound weakly to the Mono-Q column leading to early elution in the gradient. Additionally, the purification (fold) of alginate lyase from all three strains, after passing through anion exchange chromatography, was approximately three times higher than that of alginate lyase after precipitation

by ammonium sulphate (Table 4.1). It can therefore be said that this step was significant in enriching the purity of the enzymes.

The fractions purified by size exclusion chromatography were pooled, concentrated using an ultrafiltration membrane (Amicon Ultra-15, 10 kDa, US) and stored at -20^{o}C for further process.

4.3.3 Characterisation of enzymes

4.3.3.1 Molecular weight of enzymes

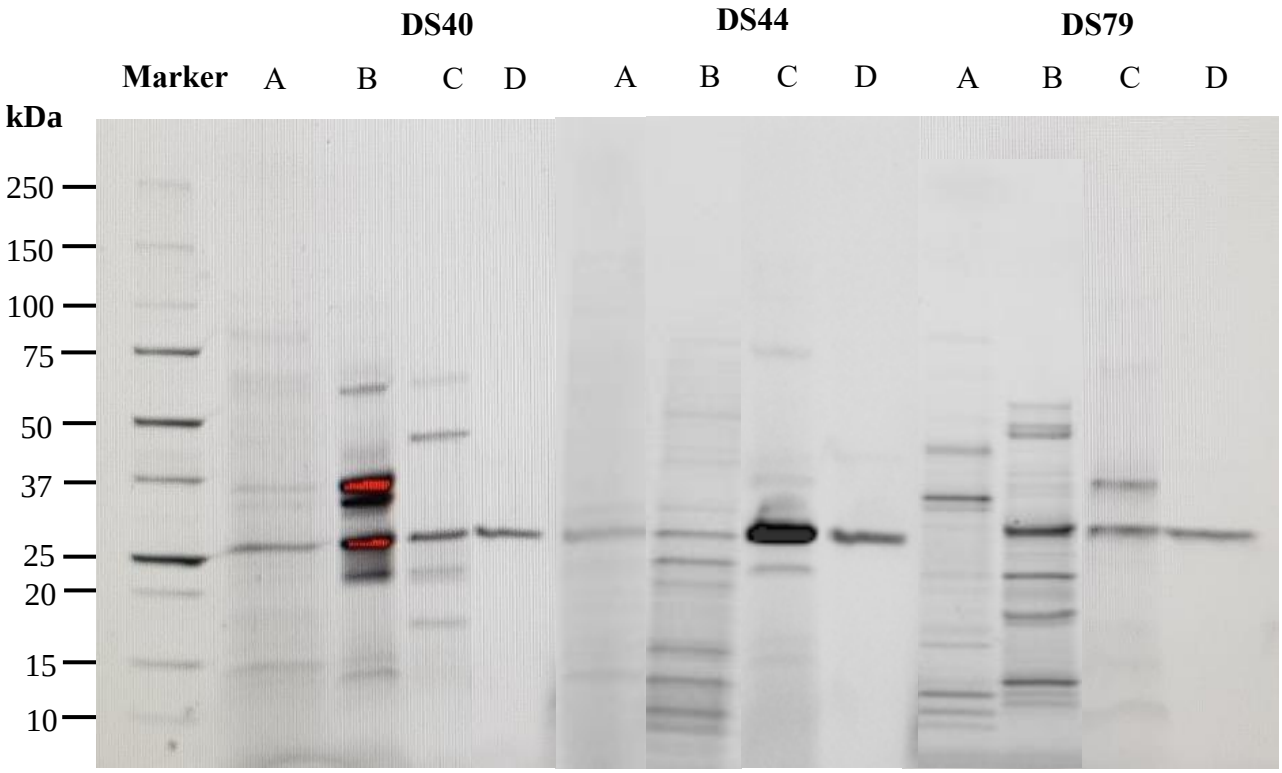


Figure 4.4: SDS – PAGE of various fractions obtained during purification: crude enzymes (A), dialysed enzymes (precipitated by ammonium sulphate) (B) and active fractions of purified enzyme from strains DS40, DS44 and DS79 by anion exchange chromatography (C) and size exclusion chromatography (D).

The fraction from strain DS40 (fraction 5) of anion exchange chromatography showed the highest specific enzyme activity toward alginate of 45.75 U/mg,

consisting of two major proteins with molecular weights of around 29 kDa and 45 kDa according to SDS-PAGE, while alginate lyase from a peak fraction of size exclusion chromatography was 67.75 U/mg showing a distinct protein band of aproximately 29 kDa (Figure 4.4). From these results, it can be confirmed that alginate lyase was pure after running size exclusion chromatography resulting in a molecular weight of approximately 29 kDa. Similarly, after the final procedure, a single band with an approximate molecular mass of 29 kDa was obtained from fractions of purified enzymes extracted from strains DS44 and DS79. These enzymes showed high specific enzyme activity as alginate lyase, accounting for 108.6 U/mg (DS44) and 103.4 U/mg (DS79). It can be concluded that all three strains can produce alginate lyase with similar molecular weight when cultured in seaweed medium. There are different sizes ranging from 24 kDa to 110 kDa from different alginate lyases produced by marine bacteria (Wong *et al.*, 2000). The DS40, DS44 and DS79 alginate lyase belong to the low molecular weight group of 25-30 kDa (Zhu & Yin , 2015). Similar molecular weights were found in alginate lyase from *Microbulbifer* sp. ALW1 (26.2 kDa) (Zhu *et al.*, 2016), *Isoptericola halotolerans* CGMCC 5336 (28 kDa) (Dou *et al.*, 2013), *Streptomyces*. sp AGL-5 (27.5 kDa) (Kim *et al.*, 2009). The other research found alginate lyase with higher molecular weights including AlyM from *Microbulbifer* sp.Q7 (63 kDa) (Yang *et al.*, 2018), AlyA5 from *Zobellia galactanivorans* (69.5 kDa) (Thomas *et al.*, 2013) and AlgH1 from *Marinimicrobium* sp. H1 (61.3 kDa) (Yan *et al.*, 2019).

In the present study, the fractions purified by size exclusion chromatography were found to be suitable for the biochemical characterisation of enzymes from strains DS40, DS44 and DS79.

4.3.3.2 Amino acid sequences and gene sequences of enzymes

The amino acid sequences of polysaccharide-degrading enzymes were identified by using 5600+ qTOF mass spectrometry (AB Sciex, Framingham, MA, USA) fitted with an Ekspert nano LC 415 high performance liquid chromatography (HPLC) (Eksigent, AB Sciex). The peptide sequences of alginate lyase from the three strains are summarised in Table 4.2, 4.3 and 4.4.

Table 4.2: Peptide summary of alginate degrading enzyme from strain DS40.

Accession numbers	Names	Strains	Sequence	Confidence	Prec MW	Theor MW	Prec m/z	Theor m/z
C0KWY1_9ACTN	*Alginate lyase*	*Streptomyces sp. M3*	AAPCDYPAQQLN	99	1346.593	1346.593	674.3036	674.3035
C0KWY1_9ACTN	*Alginate lyase*	*Streptomyces sp. M3*	FVVSGGQIK	99	933.5275	933.5284	467.771	467.7715
C0KWY1_9ACTN	*Alginate lyase*	*Streptomyces sp. M3*	KVTLPTGSSGSPTEVK	99	1586.849	1586.852	529.9571	529.9578
C0KWY1_9ACTN	*Alginate lyase*	*Streptomyces sp. M3*	LEGTSLYITKGD	99	1295.663	1295.661	648.8389	648.8377
C0KWY1_9ACTN	*Alginate lyase*	*Streptomyces sp. M3*	LPNDKPHVVGAQIHDGDDDVTVFR	99	2643.31	2643.304	661.8347	661.8333
C0KWY1_9ACTN	*Alginate lyase*	*Streptomyces sp. M3*	NASWSATSGTHTMTFR	99	1769.777	1769.779	590.9328	590.9337
C0KWY1_9ACTN	*Alginate lyase*	*Streptomyces sp. M3*	SAVNAVTTPNSSYGR	99	1522.731	1522.738	762.373	762.3761
C0KWY1_9ACTN	*Alginate lyase*	*Streptomyces sp. M3*	SSSPWFTVNSK	99	1238.592	1238.593	620.3033	620.3038
C0KWY1_9ACTN	*Alginate lyase*	*Streptomyces sp. M3*	VTLPTGSSGSPTEVK	99	1458.756	1458.757	730.3851	730.3856
C0KWY1_9ACTN	*Alginate lyase*	*Streptomyces sp. M3*	EAFNKLPNDK	98.83	1046.503	1046.503	524.2587	524.2589

Table 4.3: Peptide summary of alginate degrading enzyme from strain DS44.

Accession numbers	Names	Strains	Sequence	Confidence	Prec MW	Theor MW	Prec m/z	Theor m/z
A0A1D2IHI3_9ACTN	*Alginate lyase*	*Streptomyces sp. AVP053U2*	FVVSGGQIK	99	933.5167	933.5284	467.7656	467.7715
A0A1D2IHI3_9ACTN	*Alginate lyase*	*Streptomyces sp. AVP053U2*	IAHTASGNYFK	99	1207.569	1207.599	403.5303	403.5401
A0A1D2IHI3_9ACTN	*Alginate lyase*	*Streptomyces sp. AVP053U2*	LEGTSLYITK	99	1123.613	1123.613	562.8137	562.8135
A0A1D2IHI3_9ACTN	*Alginate lyase*	*Streptomyces sp. AVP053U2*	NASWSATSGTH	99	1117.478	1117.479	559.7463	559.7467
A0A1D2IHI3_9ACTN	*Alginate lyase*	*Streptomyces sp. AVP053U2*	SAVNAVTTPNSSYGR	99	1522.767	1522.738	762.3909	762.3761
A0A1D2IHI3_9ACTN	*Alginate lyase*	*Streptomyces sp. AVP053U2*	SSSNYGQVSLYK	99	1331.65	1331.636	666.8322	666.8251
A0A1D2IHI3_9ACTN	*Alginate lyase*	*Streptomyces sp. AVP053U2*	TSGTHTLTFR	99	1119.567	1119.567	560.7909	560.7909
A0A1D2IHI3_9ACTN	*Alginate lyase*	*Streptomyces sp. AVP053U2*	VTLPTGSSGSPTEVK	99	1458.784	1458.757	730.3992	730.3856
A0A1D2IHI3_9ACTN	*Alginate lyase*	*Streptomyces sp. AVP053U2*	VVGAQIHDGDDDVTVFR	99	1841.906	1841.891	614.9758	614.9709
A0A1D2IHI3_9ACTN	*Alginate lyase*	*Streptomyces sp. AVP053U2*	VYYNGVLQTTIAH	99	1478.772	1478.741	740.3932	740.3776

Table 4.4: Peptide summary of alginate degrading enzyme from strain DS79.

Accession numbers	Names	Strains	Sequence	Confidence	Prec MW	Theor MW	Prec m/z	Theor m/z
C0KWY1_9ACTN	*Alginate lyase*	*Streptomyces sp. M3*	AGGYTQANCGNSSPC	99	1542.578	1542.583	772.2962	772.2986
C0KWY1_9ACTN	*Alginate lyase*	*Streptomyces sp. M3*	CGNSSPCSSSNYGQVTIYK	99	2107.895	2107.894	1054.955	1054.954
C0KWY1_9ACTN	*Alginate lyase*	*Streptomyces sp. M3*	LPNDKPHVVGAQIHDGDDDVTVFR	99	2643.302	2643.304	529.6678	529.6681
C0KWY1_9ACTN	*Alginate lyase*	*Streptomyces sp. M3*	EAFNKLPNDKPHVVGAQIHDGD	99	2515.206	2515.209	629.8087	629.8096
C0KWY1_9ACTN	*Alginate lyase*	*Streptomyces sp. M3*	NASWSATSGTHTMTFR	99	1769.773	1769.779	590.9315	590.9337
C0KWY1_9ACTN	*Alginate lyase*	*Streptomyces sp. M3*	TSGTHTMTFR	99	1137.516	1137.524	569.7653	569.7691
C0KWY1_9ACTN	*Alginate lyase*	*Streptomyces sp. M3*	SGTHTMTFR	99	1036.472	1036.476	519.2434	519.2453
C0KWY1_9ACTN	*Alginate lyase*	*Streptomyces sp. M3*	AAPCDYPAQQLN	98.23	1295.588	1295.593	432.8699	432.8716
A7LFP7_9ACTN	*Alginate lyase*	*Streptomyces sp. ALG5*	AGGYTQANCGNSSPC	99	1542.578	1542.583	772.2962	772.2986
A7LFP7_9ACTN	*Alginate lyase*	*Streptomyces sp. ALG5*	IAHTASGNYFK	99	1207.593	1207.599	403.5384	403.5401

Strains DS40, DS44 and DS79 were sent to The United States Department of Agriculture (Peoria, IL, United States) to analyse the whole genome. The results were showed in Table 4.5:

Table 4.5: The whole genome analysis of strains DS40, DS44 and DS79.

Strain name	Size (Mbp)	GC content	Genera/Species ID	Identity
DS40	11.02	64.2%	*Streptomyces griseorubens*	100.0%
DS44	13.28	67.6%	*Streptomyces luridiscabiei*	99.9%
DS79	6.65	71.4%	*Streptomyces sundarbansensis*	99.9%

Some peptide sequences have around 12-26 amino acid from mass spectrometry results were convert to DNA sequence by using codon usage table for the rich G+C *Streptomyces* (Wright & Bibb, 1992). The alginate lyase gene was found by comparing the longest DNA sequence (18-22 nucleotides) with the whole genome using the Geneious software. The gene sequences of alginate lyase from three strains were showed as below:

> DS40 alginate lyase gene

ATGAGCCGCACCCGCAAGCGCACTCTCGCCACCACCGGCGTGGCCGCACTC
TCCGCCCTGGCCGCCCTGACCCTCCCGCTCACCACTGCGGGCACCGCCACC
GCGGCCGCCCCGTGCGACTACCCCGCCCAGCAGCTCAACCTGACCAACTGG
AAGGTAACCCTGCCGACCGGCTCCAGCGGCTCGCCCACCGAGGTCAAGCAG
CCGGCTCTCGCGACCTTCTCGTCCAGCCCCTGGTTCACCGTGAACTCCAAGT
GCACCGGCGTCCAGTTCCGGTCGGCGGTCAACGCCGTCACGACACCCAACT
CCAGCTACGGACGCGCCGAGCTGCGTGAGATGACCGACAACGGAACCAAGA
ACGCCTCCTGGTCGGCGACGTCCGGCACCCACACCATGACCTTCCGGGAGG
CGTTCAACAAGCTCCCCAACGACAAGCCGCACGTGGTCGGCGCCCAGATCC

ACGACGGCGACGACGACGTGACGGTCTTCCGCCTGGAGGGGACCAGCCTCT
ACATCACCAAGGGCGACGACACCCACCACAAGCTCGTGACCAGCAACTACAA
GCTGCACACGGTCTTCGAGGGCAAGTTCGTCGTCAGCGGCGGCCAGATCAA
GGTGTACTACAACGGCGTCCTCCAGACCACGATCCCGCACACCGCGTCCGG
CAACTACTTCAAGGCCGGCGGCTACACCCAGGCCAACTGCGGCAACTCCTC
CCCGTGCAGCAGCTCCAACTACGGTCAGGTGAGCATGTACAAGCTCCAGGT
CACCCACGCCTGA

> DS44 alginate lyase gene

ATGCGTCTCACTCGCAAGCGCACGCTCACCACGGCCGGCGTGGCCGCACTG
TCCGCCCTCGCGGCACTGACCGCGCCCGTCGTCGCCTCCGGCACCGCGGC
CGCGGCCGCCCCCTGCGACTACCCGGCCCAGAAGCTCAACCTCACCAACTG
GAAGGAGACCCTGCCGACCGGGTCCTCCGGCTCGCCGACCGAGATCAAGCA
GCCCGCACTCGCCACCTACTCCTCGAACCCGTGGTTCATGGTGAACGCCGC
GTGCACGGGAGTGCAGTTCCGCTCCCCGGTCAACGGCGTGACCACCTCCGG
CTCCAGCTACGCGCGCTCCGAACTGCGCGAGATGACGAGCAACGGCACGAA
GAACGCCTCCTGGTCGGCGACCTCGGGCACCCACACCCTCACCTTCCGGGA
AGCCTTCAACAAGCTCCCGAGCACCAAGCCGCACGTCGTCGGCGCGCAGAT
CCACGACGGCGACGACGACGTCACGGTCTTCCGCCTCGAAGGAACCAGCCT
CTACATCACCAAGGGTGACACCACCCACCACAAGCTCGTGACCAGCAACTAC
AAGCTGAACACGGTCTTCGAGGGCAAGTTCGTCGTCAGCGGCGGCCAGATC
AAGGTGTACTACAACGGCGTCCTCCAGACGACCATCGCGCACACGGCCTCC
GGCAACTACTTCAAGGCCGGCGCGTACACCCAGGCCAACTGCGACAACTCC
TCCCCGTGCAGCAGCTCCAACTACGGCCAGGTGAGCATCTACAAGCTGGAG
GTCACCCACTCCTGA

> DS79 alginate lyase gene

ATGAGCCGCACCCGCAAGCGCACTCTCGCCACCACCGGAGTGGCCGCACTC
TCCGCCCTGGCCGCCCTGACCCTCCCGCTCGTCACGGGGGGGCACCGCCAC
CGCGGCCGCCCCGTGCGACTACCCCGCCCAGCAGCTCAACCTGACCAACTG
GAAGGTCACCCTGCCGACCGGCTCCAGCGGCTCGCCCACCGAGGTCAAGCA
GCCCGCTCTCGCGACCTTCTCGTCCAGCCCCTGGTTCACGGTGAACTCCAAG
TGCACCGGCGTCCAGTTCCGGTCGGCGGTCAACGCCGTCACGACACCCAAC
TCCAGCTACGGACGCGCCGAGCTGCGCGAGATGACCGACAACGGAACCAAG
AACGCCTCCTGGTCGGCGACGTCCGGCACCCACACCATGACCTTCCGGGAG
GCGTTCAACAAGCTGCCCAACGACAAGCCGCACGTCGTCGGCGCGCAGATC
CACGACGGCGACGACGACGTCACCGTCTTCCGTCTGGAAGGCACCAGCCTC
TACATCACCAAGGGCGACAACACCCACCACAAGCTGGTGACCAGCAACTACC
AGCTGAACACGGTCTTCGAGGGCAAGTTCGTCGTCAGCGGCGGCCAGATCA
AGGTGTACTACAACGGCGTCCTTCAGACGACCATCGCGCACACCGCGTCCG
GGAACTACTTCAAGGCCGGCGGCTACACCCAGGCCAACTGCGGCAACTCCT
CCCCGTGCAGCAGCTCCAACTACGGTCAGGTGACCATCTACAAGCTCCAGGT
CACCCACGCCTGA

After getting the full alginate lyase gene sequences, the full amino acid sequences were found by using translation tool ExPASy (Expert Protein Analysis Systerm) to convert DNA to protein as following:

> DS40 alginate lyase protein
MSRTRKRTLATTGVAALSALAALTLPLTTAGTATAAAPCDYPAQQLNLTNWKVTL
PTGSSGSPTEVKQPALATFSSSPWFTVNSKCTGVQFRSAVNAVTTPNSSYGRA
ELREMTDNGTKNASWSATSGTHTMTFREAFNKLPNDKPHVVGAQIHDGDDDVT
VFRLEGTSLYITKGDDTHHKLVTSNYKLHTVFEGKFVVSGGQIKVYYNGVLQTTIP
HTASGNYFKAGGYTQANCGNSSPCSSSNYGQVSMYKLQVTHA

> DS44 alginate lyase protein
MRLTRKRTLTTAGVAALSALAALTAPVVASGTAAAAAPCDYPAQKLNLTNWKETL
PTGSSGSPTEIKQPALATYSSNPWFMVNAACTGVQFRSPVNGVTTSGSSYARS
ELREMTSNGTKNASWSATSGTHTLTFREAFNKLPSTKPHVVGAQIHDGDDDVTV
FRLEGTSLYITKGDTTHHKLVTSNYKLNTVFEGKFVVSGGQIKVYYNGVLQTTIAH
TASGNYFKAGAYTQANCDNSSPCSSSNYGQVSIYKLEVTHS

> DS79 alginate lyase protein
MSRTRKRTLATTGVAALSALAALTLPLVTGGTATAAAPCDYPAQQLNLTNWKVTL
PTGSSGSPTEVKQPALATFSSSPWFTVNSKCTGVQFRSAVNAVTTPNSSYGRA
ELREMTDNGTKNASWSATSGTHTMTFREAFNKLPNDKPHVVGAQIHDGDDDVT
VFRLEGTSLYITKGDNTHHKLVTSNYQLNTVFEGKFVVSGGQIKVYYNGVLQTTIA
HTASGNYFKAGGYTQANCGNSSPCSSSNYGQVTIYKLQVTHA

From all above results, alginate lyase genes of strains DS40, DS44, and DS79 were composed of 780 bp encoding 259 amino acid residues. It can be seen that strains DS40 and DS79 have the same amino acid sequence of alginate lyase leading to the same type of alginate lyase. Therefore, strains DS44 and DS79 were chosen for further characterization of alginate lyase.

4.3.3.3 Multiple sequence alignment

```
A9mT          ------------------------------------------------------------  0
AlyVOA        ------------------------------------------------------------  0
AlyVI         ------------------------------------------------------------  0
ALGNJU-03     MKSKLVNIVGSAVLLSSFAAHSAEVNLVNPSFEQDFSGWTEVDPTAVSGVAYDGAKSAKF  60
DS44          ------------------------------------------------------------  0
ALG5          ------------------------------------------------------------  0
DS79          ------------------------------------------------------------  0

A9mT          -----------------------------------------------------MTLRKTK  7
AlyVOA        -----------------------------------------------------MIKSN  5
AlyVI         ------------------------------------------------MKTSWFIDKVCSP--F  14
ALGNJU-03     SGNGARLEQSVPVTSNTEYTLSAYVLADANIGVEVGSDTFSKTASNSDWAQTTITFNSGD  120
DS44          -----------------------------------------------------MRLTR  5
ALG5          -----------------------------------------------------MTRTR  5
DS79          -----------------------------------------------------MSRTR  5

A9mT          ------------MLGLTT-ALLLSSQAFAM-TFNDAGDNRGIPADYAQYRPILSESELQ  52
AlyVOA        ------------LVVSGL-ALMSSMSYAGV-EFSNPSGQLGEPANYSQFANILSASELQ  50
AlyVI         VLQIIFMFNYQQPLKYLKVAAFISSGLLLAGCEANAK-SEQAELKTCTDCNW--NIEQWK  71
ALGNJU-03     ATEITIFGEYSGAEGRVDLFKLTSSEIIDPP-TTSLP-VFDLDPALPPSGNF--DLLDWK  176
DS44          KRTLT-------TAGVAALSAL--AALTAPV-VASGT-AAAAAPCDYPAQKL--NLTNWK  52
ALG5          KRTLA-------TTGVAALSAL--AALTLPL-VTGGT-ATAAAPCDYPAQQL--NLTNWK  52
DS79          KRTLA-------TTGVAALSAL--AALTLPL-VTGGT-ATAAAPCDYPAQQL--NLTNWK  52
                                    :  :        .                 .  : :

A9mT          ISDPAGKKGN-----------------KLYIATNTDFSGVVNEVFYVDKDTEAF--IFK  92
AlyVOA        ISDPNGKKGN-----------------KEYFGLDNDFTGIVNDNFYVDKQSQAL--VFK  90
AlyVI         ITLPVSGDDYYNNGRSSAAELIPQECNGKEYLTN-----DTHLPWFSRESVNGEERLKFT  126
ALGNJU-03     LDLPVDDNGNASGDA---QE-----VK-EGELSS-----GFENSEFFYTGDDGGL--VFI  220
DS44          ETLPTGSSGSP-------TE-----IK-QPALAT-----YSSNPWFMVNAACTGV--QFR  92
ALG5          VTLPTGSSGSP-------TE-----VK-QPALAT-----FSSRPWFTVNSKCTGV--QFR  92
DS79          VTLPTGSSGSP-------TE-----VK-QPALAT-----FSSSPWFTVNSKCTGV--QFR  92
                 * . ..                  :  :           *           *

A9mT          M----------TGDHKRNELRVHKNFNTSLPHTFYHLNASIEPVN---------------  127
AlyVOA        I----------ANDHLRNELRVQKNFRTDLPDHFYTLNANVEIMH---------------  125
AlyVI         VDLGGQVSTTANTKYARSELRELYKFNTENRCS--TKDQNWAVTG-THEL----------  173
ALGNJU-03     SPV-EGATTSANTKYTRSEMREMLRRGDTSISTTGITKNNWVFASAPSDDQNNSGGVDGV  279
DS44          SPV-NGVTT-SGSSYARSELREMTSNGTKNAS--------WSATSGTHTL----------  132
ALG5          SAV-NAVTT-PNSSYGRAELREMTDNGTKNAS--------WSATSGTHTM----------  132
DS79          SAV-NAVTT-PNSSYGRAELREMTDNGTKNAS--------WSATSGTHTM----------  132
                        .: * *:*
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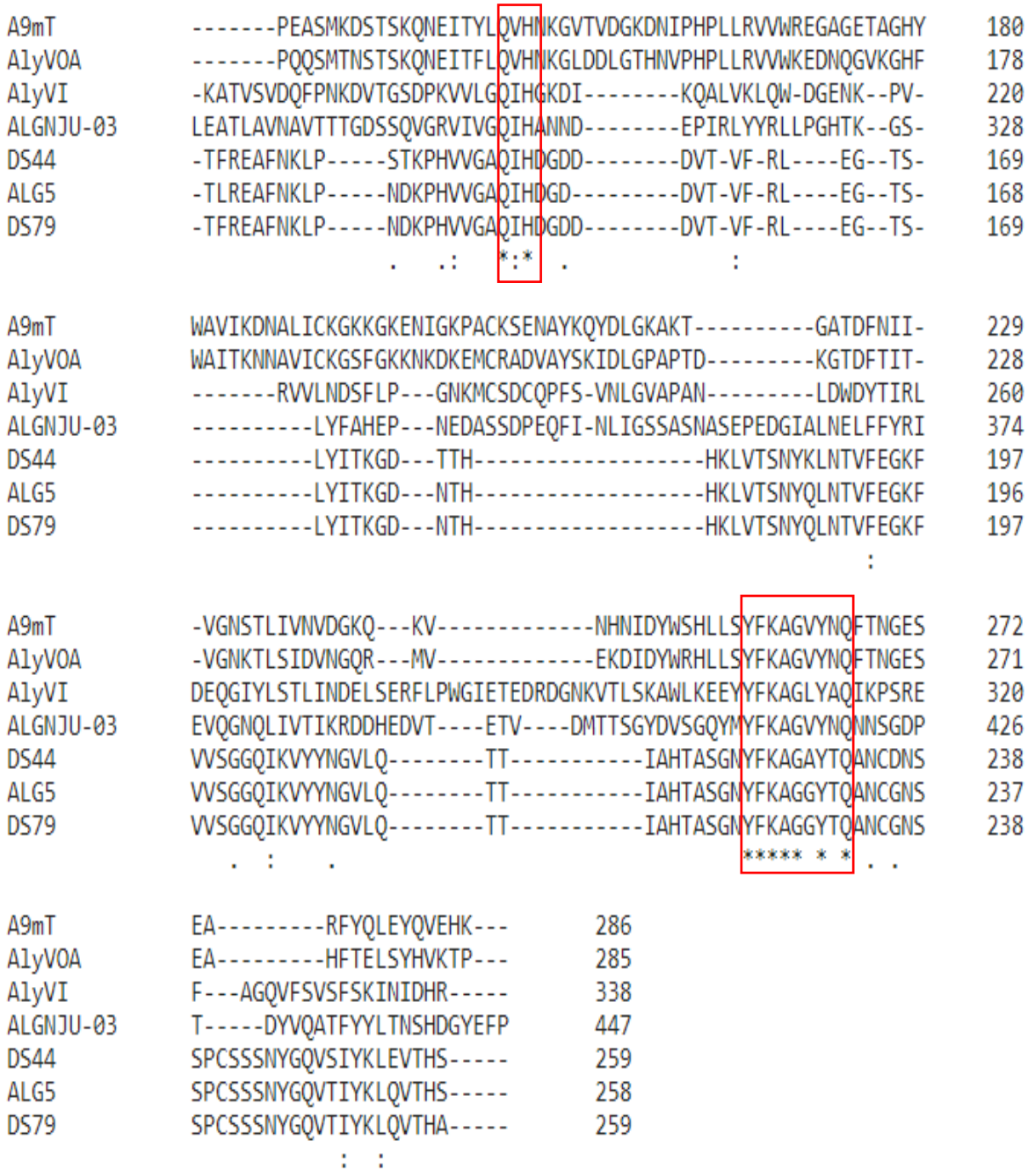

Figure 4.5: Multiple sequences alignments of amino acid sequences of DS44 and DS79 alginate lyases with other alginate lyases. Five alginate lyases: AlyVI from *Vibrio* sp. QY101 (AAP45155.1), AlyVOA from *Vibrio* sp. O2 (ABB36771.1), A9mT from *Vibrio* sp. A9m (BAH79132.1), AlgNJU-03 of *Vibrio* sp. NJU-03 (ASA33933.1) and ALG-5 from *Streptomyces*. sp ALG5 (ABS59291.1) were used for comparisons with DS44 and DS79 alginate lyase. Symbol (*) below the sequences presumed to form an active centre.

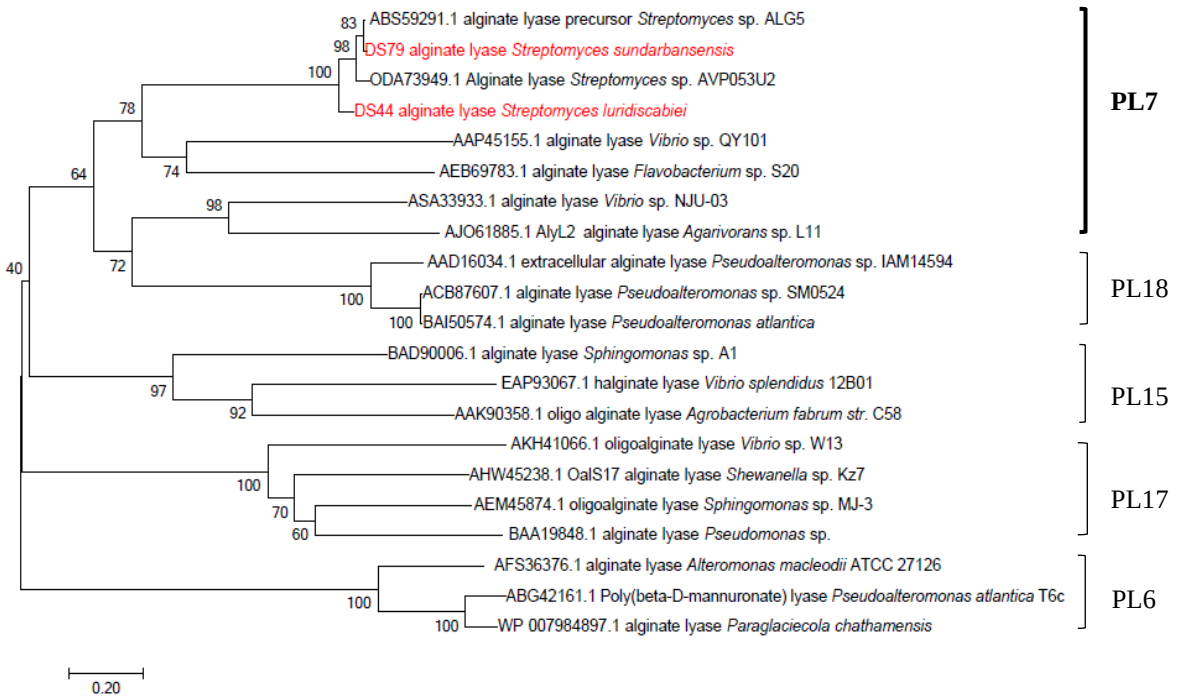

Figure 4.6: Phylogenetic tree is built from the sequence of DS44 and DS79 alginate lyases with other reported alginate lyases. Phylogenetic tree was generated by using the Neighbor-Joining method in Mega7 software. Genbank accession numbers of alginate lyase sequences and species names were given. The relationship between DS44 and DS79 alginate lyase and different PL families including PL6, PL7, PL15, PL17 and PL18 was indicated. The number at the branching points are the percentages of occurrence in 1000 bootstrapped trees. The bar indicates a distance of 0.2 substitutions per site.

From the review, alginate lyases are classified into Polysaccharide Lyase (PL) 5, 6, 7, 14, 15, 17 and 18 families based on the similarity of protein sequences and structural features (Zhu & Yin, 2015) in which PL7 family contains the largest number of members that were mostly found in bacteria (Inoue, 2018). PL7 alginate lyase consists of three highly conserved regions including R(S/N)ERL(E/A/V), Q(I/V)H and YFKAG(A/G/N/V/L)Y (Yamasaki *et al.*, 2005). Some alginate lyases of the PL7 family from *Vibrio* spp. and *Streptomyces* sp. ALG5 which have different substrate specificity were compared with the identified amino acid sequences of strains DS44 and DS79 to reveal the presence of similar sequences. The multiple sequence alignment of the catalytic domain of DS44 and DS79 alginate lyases from

Figure 4.5 indicated that they contained three conserved regions of R (S/A) ERL, QIH and YFKAG(A/G)Y. It was speculated that DS44 and DS79 alginate lyases belong to the PL7 family. For the relationship between amino acid sequence and substrate specific, Zhu & Yin (2015) found that the polyM specific alginate lyase contained QVH, while the polyG specific alginate lyase contained QIH in the conserved regions. For example, alginate lyase A9mT of *Vibrio* sp. JAM-A9m and AlyVOA of *Vibrio* sp. O2 preferred to degrade polyM, containing QVH regions (Kawamoto *et al.*, 2006; Uchimura *et al.*, 2010). AlyVI from *Vibrio* sp. QY101 and AlgNJU-03 of *Vibrio* sp. NJU-03 possessing QIH in the conserved regions were shown to have higher specific activity in polyG substrate (Han *et al.*, 2004; Zhu *et al.*, 2018). The resulted in multiple sequence alignment of the identified amino acid sequences of DS44 and DS79 showed that the enzymes having a QIH motif prefer to degrade polyG. The structural relationship between DS44, DS79 alginate lyase and other characterised alginate lyases which belong to PL6, PL7, PL15, PL17 and PL18 families was analysed and shown in the phylogenetic tree (Figure 4.6) and it also indicated that DS44 and DS79 alginate lyase are members of the PL7 family. It should be pointed out that, the amino acid sequence of DS79 alginate lyase was almost similar to the reported for the alginate lyase from *Streptomyces* sp. ALG-5, whereas DS44 alginate lyase showed more than 10% difference (Kim *et al.*, 2009) which demonstrated that DS44 alginate lyase from *Streptomyces luridiscabiei* could be a novel enzyme.

4.3.3.4 Primers for detecting alginate lyase

Primers to amplify the alginate lyase genes were designed following the comparison of sequences from multiple strains by using Geneious Prime software. There were

four possible forward primers and three possible reverse primers which were designed as shown in Table 4.6:

Table 4.6: The primers for amplifying the genes of alginate lyase.

Primer	Primer sequence (5'-3')
Forward	
H1	GCGTGGACATCAAGGAGAAC
H3	CGTSGARMRCAACGACTGYA
H5	CCAGMWSGACCCCAABT
H7	GSAGCGGCAACACVTTC
Reverse	
H2	GTGCCGGGCCTCCYAGT
H4	TGCCGGGCCTCCTYGTC
H6	AYCGGGMWAKGTGCGAATS

R = A + G, Y = C + T, M = A + C, K = G + T, S = G + C, W = A + T, B = G + T + C, V = G + A + C

After running 12 PCR samples for each strain with all the above forward and reverse primers, it was found that the primers that worked for both strains are forward primer: H1F (5'-GCGTGGACATCAAGGAGAAC-3') and reverse primer: H6R (5'-AYCGGGMWAKGTGCGAATS-3'). As can be seen in Figure 4.7, two bands with a size of 780 bp were detected on the gel, showing the results using primers for amplifying the genes of alginate lyase from the DNA of strains DS44 and DS79.

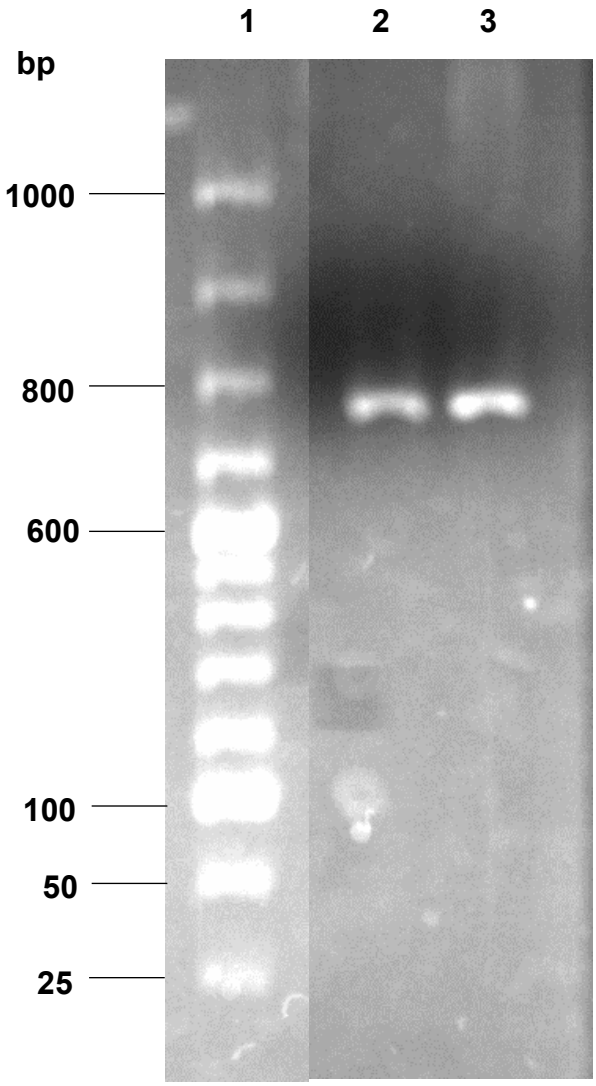

Figure 4.7: Agarose gel electrophoresis for checking the amplification product of alginate lyase genes using primers H1F and H6R. Lane 1: 1000 bp DNA ladder, lane 2: Strain DS44, lane 3: Strain DS79.

4.3.3.5 Substrate specificity of alginate lyase

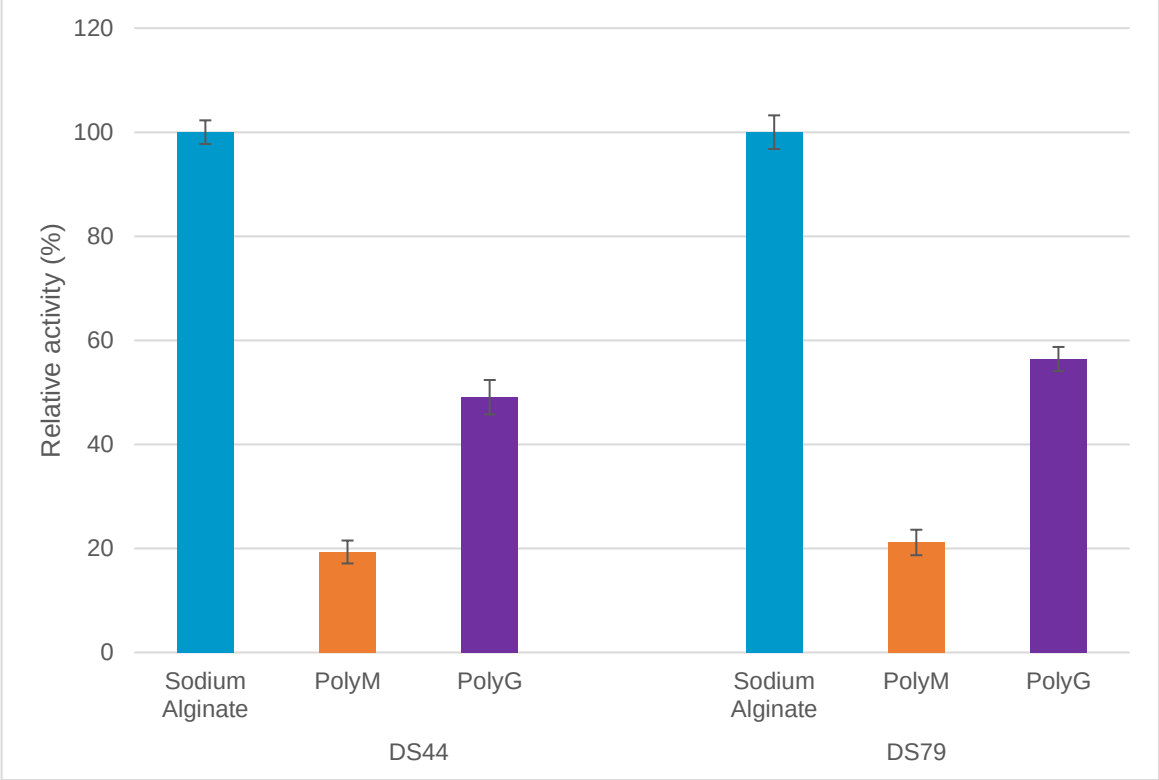

Figure 4.8: Enzyme activity of strains DS44 and DS79 grown in Bull Kelp powder medium against three different substrates (Sodium Alginate, Polymannuronic-PolyM, Polyguluronic - PolyG).

As seen in Figure 4.8, the substrate specificity of alginate lyases produced strains DS44 and DS79 growing in Bull Kelp powder medium was assessed. Alginate lyase extracted from strains DS44 and DS79 exhibited highest activity toward sodium alginate. Futhermore, they could degrade both polyM and polyG, indicating that they are bifunctional alginate lyases, however, they degraded polyG more efficiently than polyM. The results indicated that both strains produce enzymes that are classified as polyG-block specific lyase. These results are consistent with the results of multiple sequences alignment described in section 4.3.3.3. From the literature review, the large proportion of alginate lyases are analysed to have activity of polyM-specific lyase (Kim *et al.*, 2011), thus the enzymes which degrade polyG more

effectively are highly desirable for the characterisation of alginate. Furthermore, DS44 and DS79 alginate lyase showed activity towards both PolyM and PolyG substrates. This characteristic suggested that they have a good potential to produce alginate oligosaccharides with a low degree of polymerisation. The studied alginate lyases having similar substrate specificity were produced by marine *Microbulbifer* sp. ALW1 (Zhu *et al.*, 2016), marine *Vibrio* sp., NJ-04 (Zhu *et al.*, 2018) and *Marinimicrobium* sp. H1 (Yan *et al.*, 2019).

4.3.3.6 HPLC analysis of degradation products

The results of substrate specificity of alginate lyase were confirmed by the use of HPLC for analysing the change in molecular weight of oligosacharides produced by the degradation of three substrates including sodium alginate, polymannuronic - PolyM, polyguluronic – PolyG at 37^{o}C for 24 hours. For the control samples, 0.5% of sodium alginate, PolyM, and PolyG in 0.02M Tris-HCl buffer were used. The degradation ability of DS44 algiante lyase was shown in Table 4.7 and Figure 4.9. It can be seen that the control sample of sodium alginate had two main peaks in which peak 1 and peak 2 had a molecular weight of 577.8 KDa and 305 KDa, made up of 44.3% and 43.5%, respectively. Interestingly, in the first hour, the enzyme started to degrate sodium alginate to oligosacharides with smaller molecular weights of 350.6 KDa, 64.9 KDa and 15.5 KDa which accounted for 10.2%, 60.7%, and 21.8%, respectively. This demonstrates that it is possible for the degradation reaction to occur at room temperature and in a very short time (during the time to set up the HPLC machine ready to load samples). After 24 hours incubation at 37^{o}C, the degradation products which had small sizes of 18.7 KDa (15.2%), 6.6 KDa (15.3%), and 3.4 KDa (35%) were detected. These results exhibited that DS44

alginate lyase has worked effectively in sodium alginate substrate and could degrade this substrate into oligosacharides that had approximately 30 times lower molecular weight. In comparision with sodium alginate, PolyM and PolyG have smaller molecular weights of 17.7 KDa (79%) and 14 KDa (80%), respectively. At the commencement of incubation (1 hour), their chains were cut into smaler sizes of 15.8 KDa (65.5%) toward polyM and 10.9 KDa (65.8%) toward polyG. They were subsequently broken down into sizes of 9.8 KDa (54.7%) toward polyM and 3.1 KDa (51%) toward polyG after 24 hours incubation. Moreover, the smallest molecular weight of degradation products obtained from the reaction between DS44 alginate lyase and polyG substrate was 0.5 KDa which accounted for 34.5% of the total. Therefore, it can be concluded that DS44 alginate lyase presented a strong activity against alginate substrate and could degrade polyG more effectively than polyM.

Similarity, DS79 alginate lyase could degrate sodium alginate from molercular weights of 577 KDa (44.3%) and 305 KDa (43.5%) to 320.4 KDa (15.2%), 51.9 KDa (24.7%), and 13.5 KDa (31.1%) at 1 hour; and then to 43 KDa (11.3%), 6.2 KDa (25.8%), and 3.3 KDa (34.2%) after 24 hours incubation (Table 4.8). These results implied that DS79 alginate lyase had a high activity toward sodium alginate. In terms of Poly M and PolyG substrates, after 24 hours incubation, this enzyme broke down PolyM from the sizes of 17.7 KDa (79%) to 10.4 KDa (58.9%), 5.8 KDa (3.7%), and 6.4 KDa (3.7%); while it cut off PolyG from the sizes of 14.4 (80.9%) to 10.2 KDa (2.9%), 2.1 KDa (49.2%), and 0.5 KDa (24.5%). It can be seen clearly that DS79 alginate lyase could degrade both PolyM and PolyG but it preferred PolyG to PolyM.

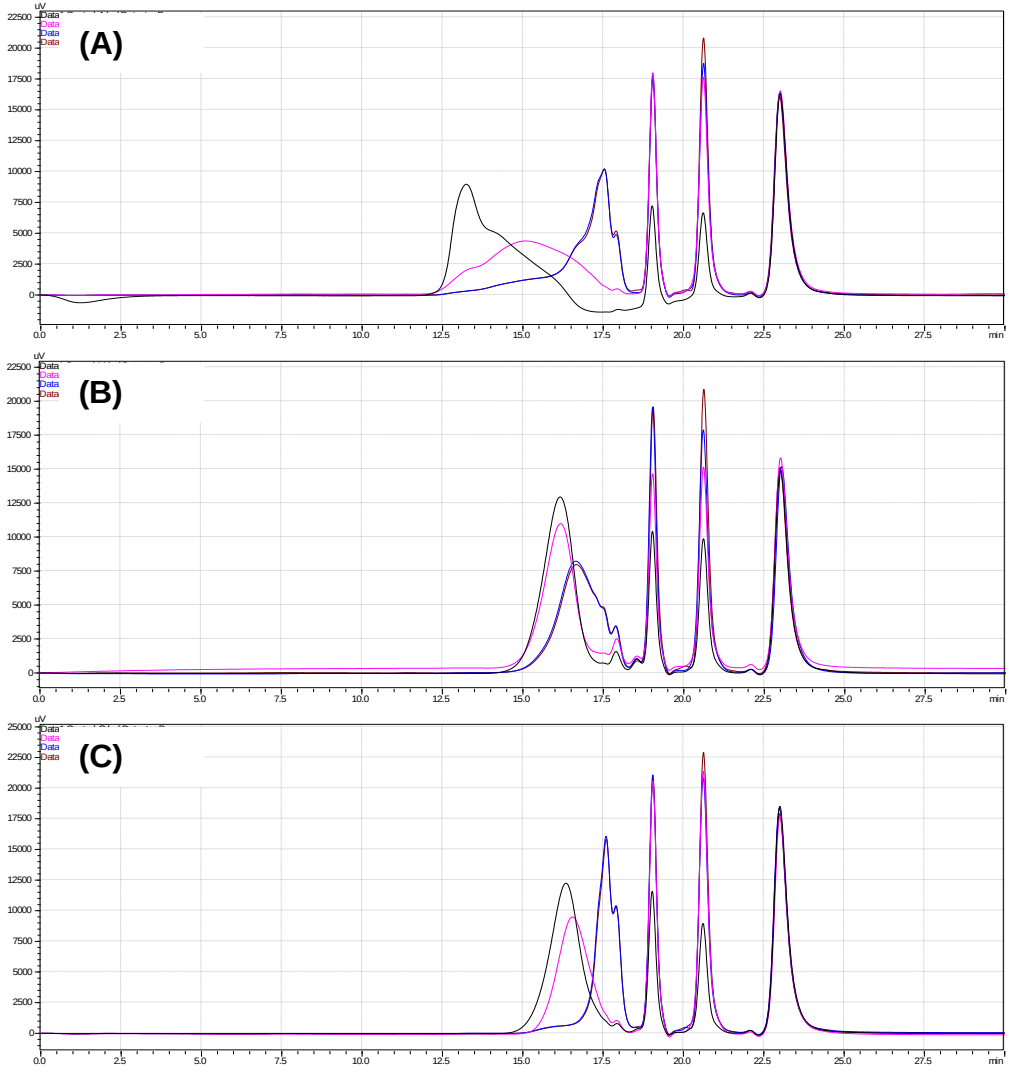

Figure 4.9: Activity of purified enzyme from strain DS44 against three substrate including sodium alginate (A), PolyM (B) and PolyG (C) were analysed by HPLC. Black line: control samples, pink line: samples at 1h incubation, blue line: samples after 24h incubation.

Table 4.7: The change of molecular weight of three substrates (Sodium alginate, PolyM and PolyG) after incubation with purified enzyme from strain DS44 at 1h and 24h.

	Peak 1		Peak 2		Peak 3	
	%	MW (kDa)	%	MW (kDa)	%	MW (kDa)
Sodium alginate						
Control	44.3	577.8	43.5	305.0	0.2	2.1
1h	10.2	350.6	60.7	64.9	21.8	15.5
24h	15.2	18.7	15.3	6.6	35.0	3.4
Poly M						
Control	79.9	17.7	2.9	2.2	1.7	1.0
1h	65.5	15.8	3.7	2.2	1.6	1.0
24h	54.7	9.8	8.3	4.1	5.8	2.2
Poly G						
Control	80.9	14.4	0.4	2.1	16.7	0.6
1h	65.8	10.9	2.5	2.1	31.9	0.5
24h	51.0	3.1	14.3	2.1	34.5	0.5

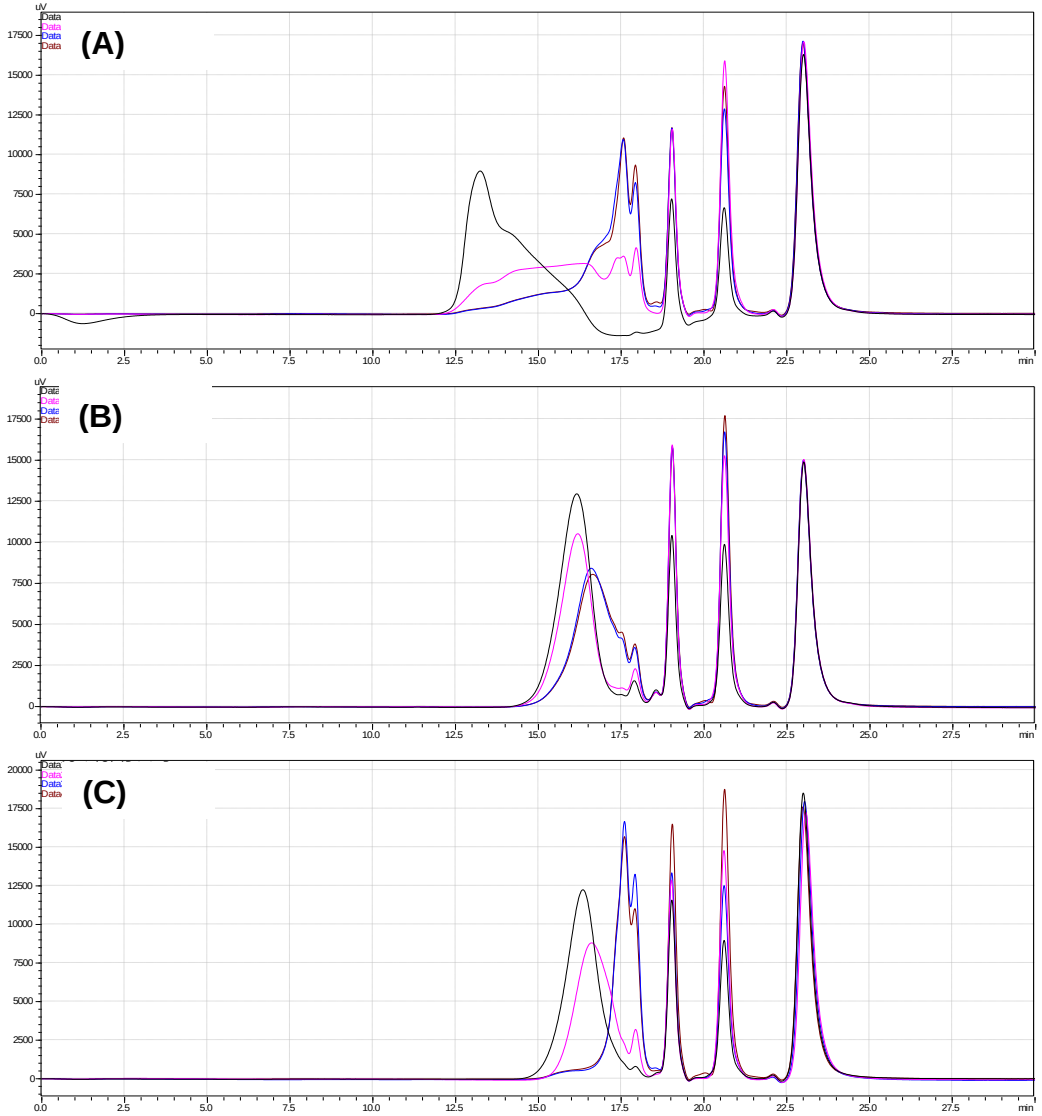

Figure 4.10: Activity of purified enzyme from strain DS79 against three substrate including sodium alginate (A), PolyM (B) and PolyG (C) were analysed by HPLC. Black line: control samples, pink line: samples at 1h incubation, blue line: samples after 24h incubation.

Table 4.8: The change of molecular weight of three substrates (Sodium alginate, PolyM and PolyG) after incubation with purified enzyme from strain DS79 at 1h and 24h.

	Peak 1		Peak 2		Peak 3	
	%	MW (kDa)	%	MW (kDa)	%	MW (kDa)
Sodium alginate						
Control	44.3	577.8	43.5	305.0	0.2	2.1
1h	15.2	320.4	24.7	51.9	31.1	13.5
24h	11.3	43.3	25.8	6.2	34.2	3.3
Poly M						
Control	79.9	17.7	2.9	2.2	1.7	1.0
1h	66.7	16.9	1.6	3.4	4.7	2.1
24h	58.9	10.4	5.8	3.7	6.4	2.1
Poly G						
Control	80.9	14.4	0.4	2.1	16.7	0.6
1h	70.0	14.4	6.5	2.1	23.5	0.6
24h	2.9	10.2	49.2	2.1	24.5	0.5

4.3.3.7 Analysis of degradation products of DS44 (A) and DS79 (B) alginate lyases by ESI-*MS*

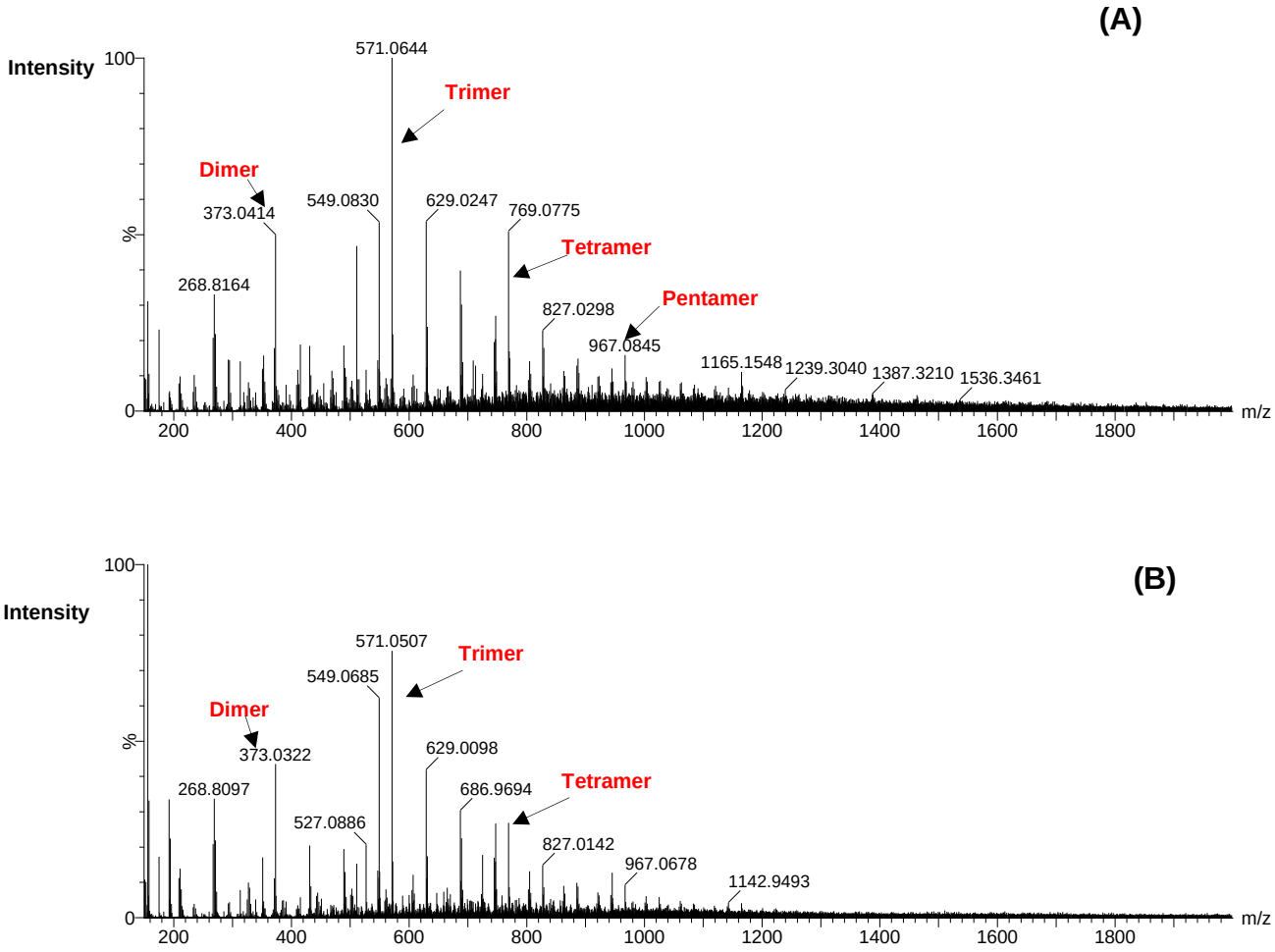

Figure 4.11: ESI-MS analysis of the hydrolysis products by DS44 (A) and DS79 (B) alginate lyases.

The negative-ion electrospray ionization mass spectra of hydrolysis products was shown in Figure 4.11. The ions at m/z 373, 571, 769, and 967 exhibited unsaturated disaccharide, unsaturated trisaccharide, unsaturated tetrasaccharide, and unsaturated pentasaccharide, respectively (Yang *et al.*, 2018). After degrading for 24 hours at 37^{o}C, the main DS44 degradation products were determined as disaccharides, trisaccharides, and tetrasaccharides; while DS79 hyrolysis products contained disaccharides and trisaccharides as the main products. The results

verified that DS44 and DS79 alginate lyases cleaved the glycosidic bonds in alginate by β-elimination reaction and also indicated that these enzymes were endo type alginate lyase (Yan *et al.*, 2019; Yang *et al.*, 2018).

4.3.3.8 The effect of pH, temperature, NaCl and metal ions on alginate lyase activity

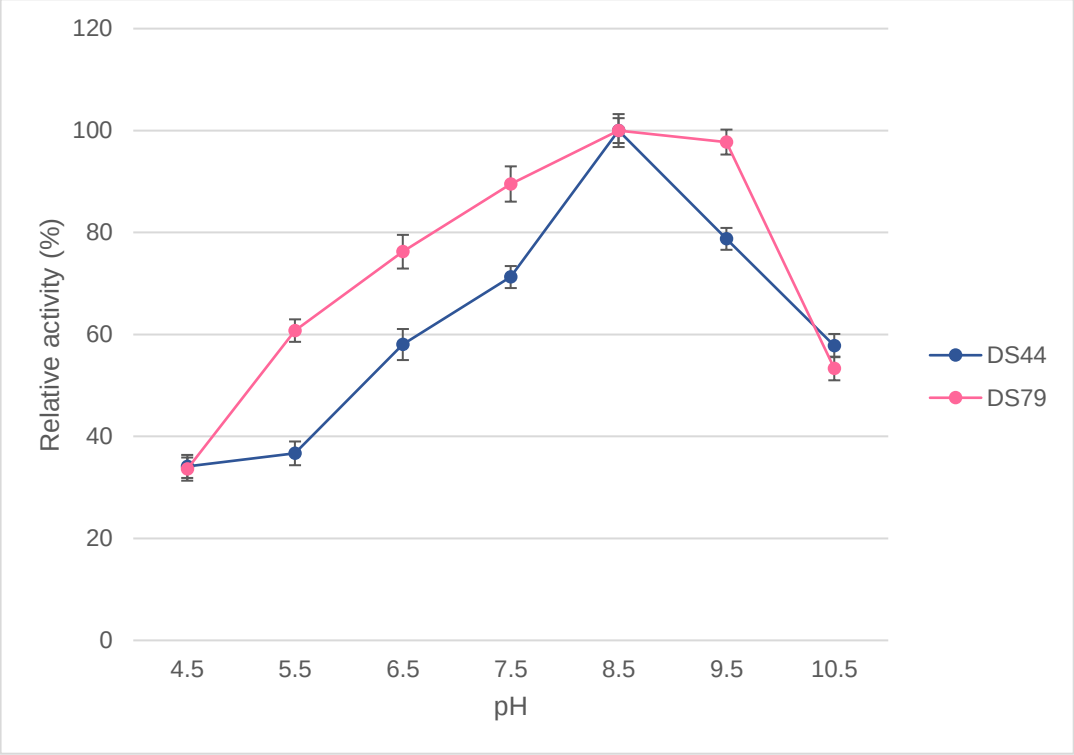

Figure 4.12: Optimal pH of DS44 and DS79 alginate lyases. The highest activity was set as 100%. Each value represented the mean of triplicates ± standard deviation.

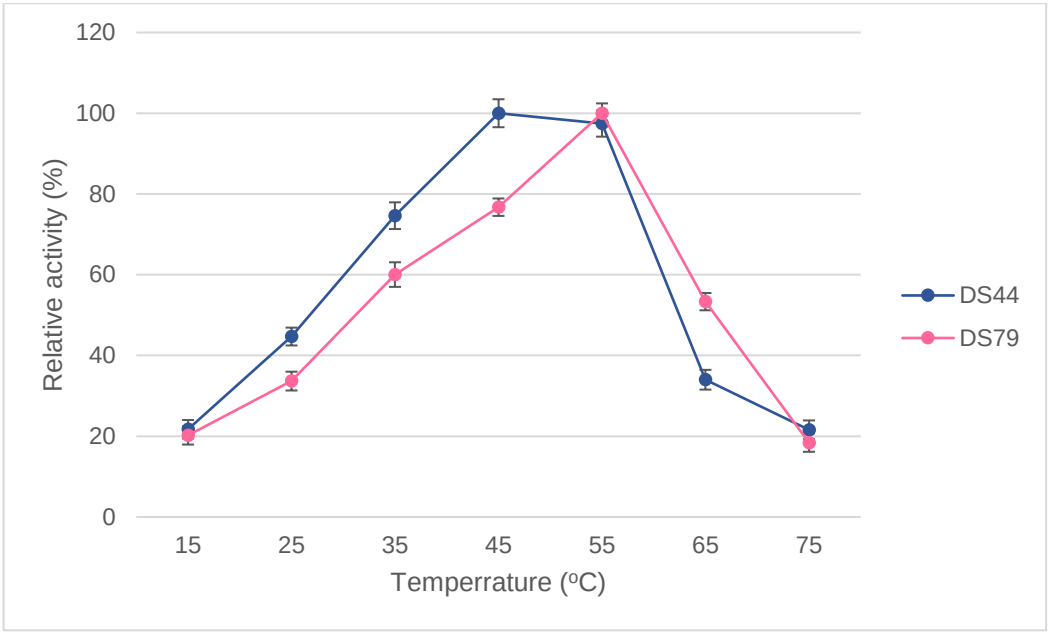

Figure 4.13: Optimal temperature of DS44 and DS79 alginate lyases. The highest activity was set as 100%. Each value represented the mean of triplicates ± standard deviation.

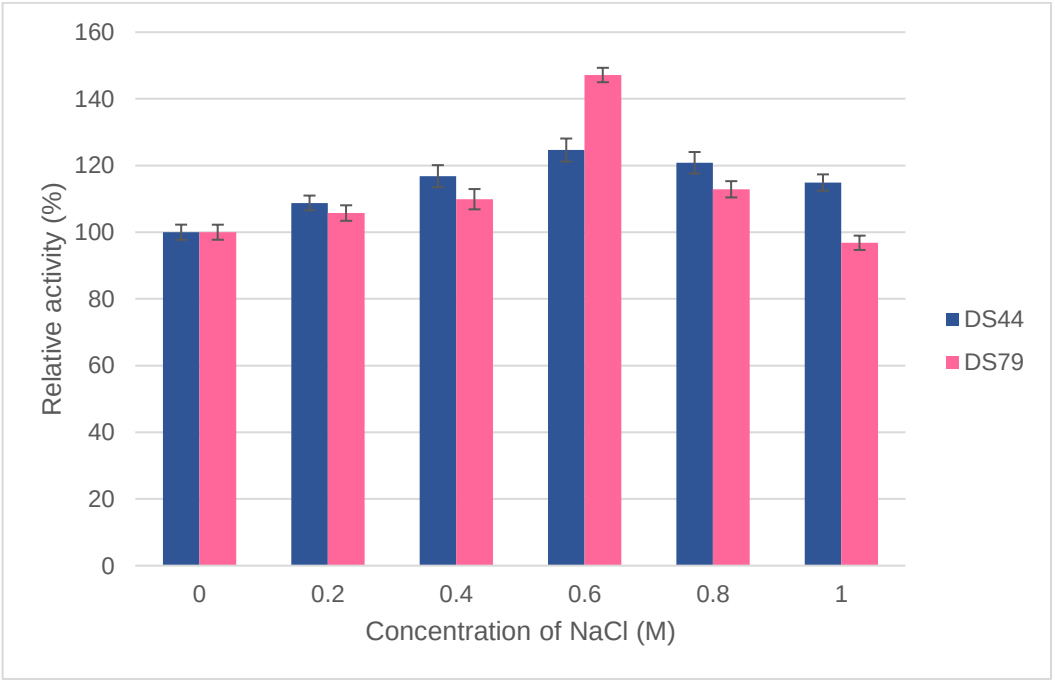

Figure 4.14: Optimal NaCl concentration of DS44 and DS79 alginate lyases. The activity of the control (no NaCl) was taken as 100%. Each value represented the mean of triplicates ± standard deviation.

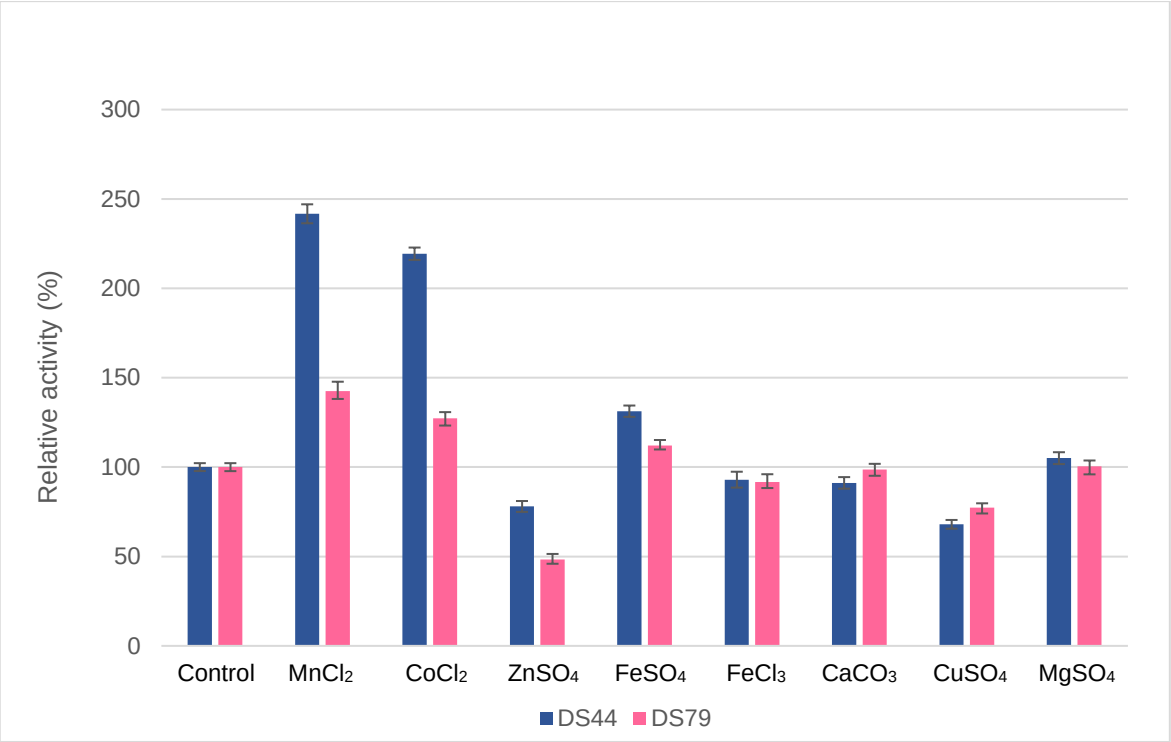

Figure 4.15: The effect of metal ions (5 mM) on the activity of DS44 and DS79 alginate lyase. The activity of the control (no metal ion) was taken as 100%. Each value represented the mean of triplicates ± standard deviation.

The optimal pH of DS44 and DS79 alginate lyse was conducted at 37°C for 2 hours over a pH range of 4.5 to 10.5. As shown in Figure 4.12, the maximum activity of both enzymes was obtained at pH 8.5 in 0.02M Tris-HCl buffer. The relative activity over 50% of its maximum activity was exhibited in the pH range of 5.5 – 10.5 and 6.5 – 10.5, according to DS79 and DS44 alginate lyase respectively. Some previous studies showed that the majority of alginate lyase from marine bacteria represented the optimal pH between 7.0 and 8.5 (Dou *et al.*, 2013). There were similar results observed in alginate lyases from this study.

The optimum reaction temperature of DS44 and DS79 alginate lyases was investigated in 0.02M Tris-HCl buffer (pH 8) at various temperatures from 15°C to 75°C for 2 hours. It can be seen in Figure 4.13, strain DS44 and DS79 had optimal

temperature of 45^{o}C and 55^{o}C, respectively. DS79 alginate lyase presented over 50% relative activity in temperature range of 35^{o}C – 65^{o}C, while relative activity of DS44 alginate lyase dropped dramatically to approximately 35% at 65^{o}C. There were significant differences in terms of optimal temperatures of the alginate lyase. The optimal temperature of enzyme isolated from strain DS44 was similar to that from *Microbulbifer* sp. ALW1, *Marinimicrobium* sp. H1, and *Shewannella* sp. YHI (Wang *et al.*, 2019; Yan *et al.*, 2019; Zhu *et al.*, 2016), but was lower than that from *Sphingomonas* sp. MJ-3 and *Microbulbifer* sp. 6532A (Park *et al.*, 2014; Wang *et al.*, 2019). Besides that, DS79 alginate lyase had an optimal temperature of 55^{o}C which was similar to AlyA lyase derived from *Isoptericola halotolerans* NJ-05 and AlyM from *Microbulbifer* sp. Q7 (Yang *et al.*, 2018; Zhu *et al.*, 2018), but higher than that from *Vibrio furnissii* H1, *Pseudoalteromonas* sp. CY24 and *Agarivorans* sp. L11 (Duan *et al.*, 2009; Li *et al.*, 2015; Zhu *et al.*, 2018).

The effect of NaCl on the enzyme activity of DS44 and DS79 alginate lyases is shown in Figure 4.14. DS44 alginate lyase was more active in the presence of a wide concentration range of NaCl (0.2 M - 1 M) and showed the highest activity of 125% with 0.6 M NaCl. A similar result was obtained by DS79 alginate lyase which exhibited the maximum activity of 147% at 0.6 M NaCl. Moverover, DS79 alginate lyase showed 22% higher activity than DS44 at the optimal concentration of NaCl. Interestingly, DS44 alginate lyase could maintain the high activity at a very high concentration of 1 M NaCl that was similar with AlyAL-28 lyase extracted from *Vibrio harveyi* AL-28 (Kitamikado *et al.*, 1992). Therefore, both enzymes in this study also belong to a group of salt-tolerant alginate lyase isolated from marine bacteria (Yan *et al.*, 2019; Zhu *et al.*, 2018; Zhu *et al.*, 2016). They are therefore potentially useful to employ in the degradation reaction with high salt substrates.

The metal ions Mn^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Ca^{2+}, Cu^{2+}, and Mg^{2+} were investigated to display their effect on the enzyme activity at a concentration of 5 mM. As shown in Figure 4.15, Mn^{2+}, Co^{2+}, and Fe^{2+} could enhance the alginate degradation activity of both enzymes. These results were similar to some reports of M3 lyase and ALG-5 alginate lyase from *Streptomyces* spp. (Kim, 2010; Shin *et al.*, 2011) and the alginate lyase from *Isoptericola halotolerans* CGMCC 5336 (Dou *et al.*, 2013). Surprisingly, DS44 alginate lyase was affected significantly by Mn^{2+} and Co^{2+} with 242% and 219% relative activity which was approximately double in comparison with the control and DS79 alginate lyase. Ca^{2+} and Mg^{2+} had no effect on the enzyme activities (Zhu *et al.*, 2016). By contrast, both enzymes were clearly inhibited by Zn^{2+} and Cu^{2+}, while Fe^{3+} showed slightly inhibitory effects (Hu *et al.*, 2019; Zhu *et al.*, 2018; Zhu *et al.*, 2016).

4.4 Conclusion

In this chapter, the purification and characterisation of DS40, DS44 and DS79 alginate lyase were accomplished. The successful purification of the alginate lyases was achieved by combining anion exchange chromatography and size exclusion chromatography. The enzymes have the molecular weight of approximately 29 KDa. The alginate lyase gene of strains DS40, DS44, and DS79 were composed of 780 bp encoding 259 amino acid residues. There was 100% similarity in the amino acid sequences of DS40 and DS79 alginate lyase leading to the same type of alginate lyase. DS44 and DS79 alginate lyase could degrade both polyM and polyG, indicating that they are bifunctional alginate lyase, however, they favourably degraded polyG more so than polyM. This characteristic suggests that they have a good potential to produce alginate oligosaccharides with low degree of

polymerisation. Moreover, based on the highly conserved regions of their amino acid sequences, these enzymes were classified to the PL7 family. The enzymes could degrade sodium alginate to produce alginate disaccharides, trisaccharides as the main end products, indicatting that these enzymes were endo type alginate lyase. Futhermore, the enzymes showed the highest activity at pH 8.5 and when the degradation reaction was performed at temperatures of 45^{o}C and 55^{o}C for DS44 and DS79 alginate lyases, respectively. They also exhibited unchanged activity at high concentration of NaCl, which led them to be grouped into salt-tolerant alginate lyases isolated from marine bacteria. Both enzymes were more active by 5 mM of Mn^{2+}, Co^{2+}, and Fe^{2+} and inhibited by Zn^{2+} and Cu^{2+} as common enzymes from marine bacteria. Besides that, the amino acid sequence of DS44 showed more than 10% difference to a reported alginate lyase from *Streptomyces* sp. ALG-5, demonstrated that DS44 alginate lyase from *Streptomyces luridiscabiei* could be novel enzyme. To sum up, DS44 and DS79 alginate lyases might be the potential industrial enzymes for efficient production of alginate oligosacharides.

On the other hand, based on the whole genome analysis, strains DS40, DS44 and DS79 showed the closest match to *Streptomyces griseorubens, Streptomyces luridiscabiei, and Streptomyces sundarbansensis,* respectively*.* Therefore, all three strains are members of the genus *Streptomyces.* sp. *Streptomyces luridiscabiei* is a known pathogen of potatoes causing scabs on the tuber (Park *et al*., 2003). Therefore, as it would not be advisable to grow this strain on a large scale, it was decided to produce this novel enzyme by recombinant means.

Table 4.9: Summary of properties of DS44 and DS79 alginate lyases.

Enzyme	Organism	Optimal pH	Optimal temperature (°C)	Molecular mass (kDa)	Substrate specificity	Action mode	Inhibitor	Activator
DS44 alginate lyase	*Streptomyces luridiscabiei*	8.5	45	29	PolyG & PolyM	Endolytic	Zn^{2+} and Cu^{2+}	Mn^{2+}, Co^{2+}, and Fe^{2+} 0.6M NaCl
DS79 alginate lyase	*Streptomyces sundarbansensis*	8.5	55	29	PolyG & PolyM	Endolytic	Zn^{2+} and Cu^{2+}	Mn^{2+}, Co^{2+}, and Fe^{2+} 0.6M NaCl

CHAPTER 5: MOLECULAR CLONING AND EXPRESSION OF ALGINATE LYASE

5.1 Introduction

Recombinant DNA technology plays an essential role in the improvement of many health conditions, enhancing food resources, product yield, and resistance to harmful agents in agriculture applications. Gene therapy and genetic modifications are extensively used for in the treatment of potentially serious diseases (Khan *et al.*, 2016). In recent years, the demand for pure bioactive proteins, such as hormones and enzymes has increased dramatically. Recombinant DNA technology has been proven to be very useful in meeting the requirement for large amounts of high purity and highly active proteins of interest (Hartley, 2006). The products of cloning and expression are commonly used in biotechnology research for antibody production, small molecule identification, structural and functional studies (Khan, 2013). For a protein to be produced recombinantly, it must have the ability to expressed and purified in a large quantity, often so that it can be used for industrial processes and the development of commercial products (Rosano & Ceccarelli, 2014). In cloning, the choice of expression vector and host cell is considered carefully. *Escherichia coli* (*E. coli*) has been the preferred microbial cell used for expression because it has significant multiple benefits over other expression systems including low costs, relatively fast growth, and ease of scale up (Baneyx, 1999; Busso *et al.*, 2005; Rosano & Ceccarelli, 2014). These advantages have allowed the affordable production of therapeutic proteins such as insulin and bovine growth hormone (Jana & Deb, 2005). Blood clotting agents and vaccines are other examples of proteins produced by recombinant methods (Khan *et al.*, 2016). To achieve a high-level expression of protein and then increase the solubility of the recombinant protein expression in *E. coli*, expression vector design, antibiotic selection, codon usage, promoter, mRNA stability, transcription termination,

translation initiation, translation signal and the culture parameters such as temperature, additive or induction conditions are the major challenges that need to be considered and modified (Busso *et al.*, 2005; Jana & Deb, 2005).

There are many previous reported on recombinant alginate lyase from various sources including AlgNJ-04 from marine *Vibrio* sp. NJ-04; ZH0-I,II,III,IV from *Sphingomonas* sp. ZH0; AlyM from *Microbulbifer* sp. Q7 and ALG-5 from *Streptomyces* sp. ALG5 which have been cloned into pET-21a (+), pGEX-4T-1, pProEX-HTa and pColdI expression vector, respectively. *E. coli* BL21 was chosen as a host for the expression of all these alginate lyase (He *et al.*, 2018; Kim *et al.*, 2009; Yang *et al.*, 2018; Zhu *et al.*, 2018). These recombinant enzymes have been promising candidates for producing alginate oligosacharides applied in industry.

In this chapter, the alginate lyase genes of strains DS44 and DS79 were cloned and heterologous expressed in an *E. coli* BL21 (DE3) expression system. The various concentrations of inducer (Isopropyl-β-D-thiogalactopyranoside - IPTG) were used to optimise the expression condition. Additionally, the target His-tagged recombinant proteins were purified by immobilized metal affinity chromatography (IMAC), using Ni-Sepharose 6 fast flow column.

5.2 Materials and methods

5.2.1 Cloning and expression alginate lyase

To clone the alginate lyase genes into an expression vector, the restriction enzymes including *Nde*1 and *Sal*1-HF (High Fidelity) (New England BioLabs) were used. The alginate lyase gene sequences were optimised for expression in *E. coli*. The DS44 and DS79 Gblock gene fragments are sequence-verified, double-stranded DNA

fragments encoding DS44 and DS79 alginate lyase genes and containing recognised sites of the restriction enzymes at the start and the end of their sequences. The Gblock gene fragments were synthesised by IDT Company. The Gblock gene fragments were ligated with pGEM-T easy vector (Promega, USA). The Gblock gene fragments and expression vector pColdI (Takara, Japan) were digested by the same restriction enzymes. Finally, the alginate lyase genes were ligated into the expression vector and then pColdI/alginate lyase gene plasmids were transformed into *E. coli* BL21 (DE3) for protein expression.

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Figure 5.1: General protocol of expression process from gene to protein (This figure has been reproduced from BiologicsCorp)**.**

> DS44 Gblock gene fragment

5'– ATCAAGTTAGAGCGGTCCACCCGTT CAT ATG ATG CGG CTG ACA AGA AAG CGT ACG TTG ACT ACG GCG GGT GTG GCT GCA CTG AGT GCT TTA GCA GCA CTG ACA GCA CCA GTA GTC GCC AGC GGG ACG GCT GCA GCT GCT GCC CCT TGC GAC TAC CCA GCA CAA AAA CTT AAC CTT ACC AAC TGG AAG GAA ACT TTG CCC ACT GGG TCA TCT GGT AGT CCT ACG GAG ATA AAA CAG CCA GCT TTG GCG ACG TAT TCG AGT AAC CCG TGG TTT ATG GTG AAT GCT GCA TGT ACT GGA GTT CAG TTT CGC TCG CCA GTA AAC GGT GTG ACC ACA TCT GGG AGC AGC TAC GCC CGG AGC GAG CTG CGT GAA ATG ACC TCT AAC GGC ACT AAG AAT GCC TCG TGG TCA GCG ACA TCC GGC ACC CAC ACA TTA ACG TTC CGT GAA GCA TTT AAT AAG CTT CCA TCC ACC AAG CCC CAC GTA GTG GGT GCG CAA ATC CAC GAC GGC GAC GAC GAC GTG ACA GTG TTC AGA TTG GAG GGA ACC AGC CTG TAT ATT ACA AAG GGG GAC ACC ACG CAC CAC AAG CTG GTA ACC TCT AAC TAC AAA CTT AAC ACA GTC TTC GAG GGC AAA TTC GTA GTT TCT GGC GGA CAA ATT AAG GTC TAT TAC AAC GGT GTT CTG CAG ACG ACT ATC GCC CAC ACT GCA TCG GGC AAT TAT TTC AAG GCC GGA GCG TAC ACC CAG GCG AAT TGT GAT AAT TCC TCA CCA TGC AGC AGC TCA AAT TAT GGA CAA GTT TCG ATA TAC AAA CTG GAA GTA ACC CAT TCA TAA AAG CTT ACGATTGCCGTCACAC –3'

> DS79 Gblock gene fragment

5'– TTTTTTCATATGATG TCC CGC ACG CGG AAA CGC ACA TTG GCC ACT ACG GGT GTT GCG GCA TTG AGT GCG CTG GCC GCT CTT ACA TTG CCA CTT GTT ACT GGT GGG ACA GCT ACT GCC GCT GCT CCG TGT GAT TAT CCT GCC CAA CAG CTG AAT CTG ACG AAC TGG AAA GTT ACC CTG CCG ACC GGG AGC AGC GGA TCT CCA ACT GAA GTG AAG CAG CCA GCT TTA GCG ACA TTT TCG TCG TCC CCA TGG TTC ACC GTG AAT AGC AAA TGC ACC GGA GTG CAG TTC AGA AGC GCA GTC AAC GCT GTC ACT ACG CCG AAT AGT TCG TAT GGT AGA GCT GAA CTG CGC GAA ATG ACT GAC AAT GGT ACA AAA AAC GCA TCA TGG TCT GCA ACA AGT GGT ACG CAT ACC ATG ACC TTC CGG GAA GCT TTT AAC AAA TTG CCT AAT GAT AAG CCT CAT GTC GTG GGC GCT CAG ATC CAC GAC GGA GAT GAT GAC GTA ACT GTC TTC CGC CTG GAG GGC ACG TCG CTT TAT ATC ACG AAA GGA GAC AAT ACT CAT CAC AAA CTG GTC ACA AGC AAC TAT CAG TTG AAC ACC GTC TTT GAG GGA AAA TTT GTA GTT TCT GGT GGC CAA ATA AAA GTT TAC TAT AAT GGG GTG CTT CAA ACA ACG ATC GCA CAC ACC GCC AGC GGT AAT TAT TTT AAA GCA GGT GGC TAT ACA CAA GCC AAT TGT GGA AAT TCC TCT CCC TGT TCG TCG TCG AAT TAC GGT CAG GTA ACC ATT TAC AAG CTT CAG GTT ACA CAT GCG TAAAAGCTTTTTTTT – 3'

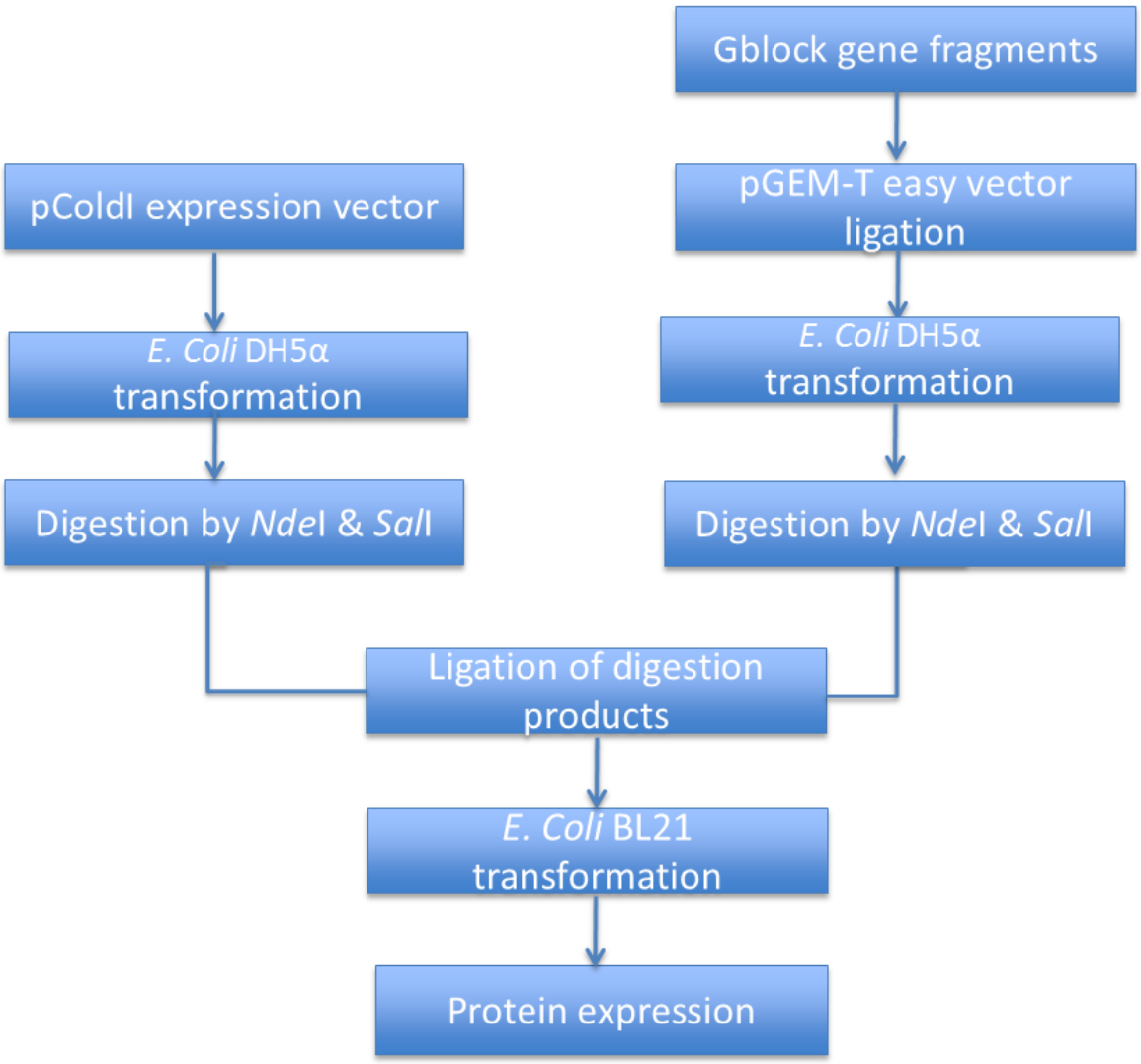


Figure 5.2: Flow diagram of cloning and expression process.

5.2.1.1 *E. coli* DH5α transformation

Expression vector pColdI (5µg/µl) was diluted to 1 ng/µl and 10 ng/µl by MQ water. 1 µl of each dilution of plasmid DNA was added to 100 µl of DH5α cells and incubated on ice for 30 minutes. The cells were heat shocked at 42°C for 2 minutes, and then incubated immediately on ice for 5 minutes. After that, the cells were left at room temperature for 5 minutes. 1 ml of LB solution (Appendix A4.1) was added to the cells followed by incubation at 37°C for 30 minutes. The transformation reaction was centrifuged at 4000 rpm for 2 minutes, and then 1ml of supernatant

was removed leaving approximately 100 µl of supernatant and the pellet of the cells. The cell were resuspended in 100 µl of supernatant and spread onto pre-warmed LB agar plates (Appendix A4.2) containing Ampicillin (100 µg/ml) to select for the cells that were successfully transformed. The plates were incubated at 37^{o}C overnight.

Poly A tailing of Gblock gene fragment: Gblock gene fragments of DS44 and DS79 were resuspended in MQ water and diluted to 10 ng/µl. The gene fragments were amplified by running amplification at 72^{o}C for 20 minutes. The amplification was carried out in 50 µl reaction volumes with the following reagents:

Component	Volume
Gblock gene fragment (100 ng/µl)	10 µl
10x Tag polymerase buffer	5 µl
10 mM dATP	1 µl
Tag polymerase	0.2 µl
Nuclease free water	33.8 µl
Total	50 µl

0.5 µl amplified Gblock gene fragments were ligated into 0.5 µl pGEM-T easy vector in 10 µl reactions with 1 µl ligase, 5 µl 2x ligase buffer and 3 µl MQ water. The ligation reaction was carried out at room temperature for 2 – 3 hours. The plasmids containing genes of interest (5 µl) were transform into DH5α cells (100 µl) by heat shock method (similar with transformation of vector pColdI) and then spread onto

pre-warmed LB agar plates containing Ampicillin (100 µg/ml) to select for the cells that were successfully transformed. The plates were incubated at 37ºC overnight.

5.2.1.2 DNA plasmid purification

Streak plates of DH5α containing pColdI vector and pGEM-T easy vector with inserts were incubated at 37ºC overnight. A single colony from the streaked plates was picked up and then inoculated in 5ml LB medium containing Ampicillin (100 µg/ml) in 100 ml Erlenmeyer flasks at 37ºC overnight with shaking. After overnight incubation, the 5ml of cultured DH5α cells were harvested by centrifugation in sterile 15 ml tubes at 4000 rpm for 5 minutes. The supernatant was gently poured off and removed completely by pipetting to maximise plasmid DNA yield. The plasmid DNA was extracted from the remaining cell pellet by using the protocol of AccuPrep® Nano-Plus Plasmid DNA Extraction Kit (Bioneer, Korea) (Appendix E1).

5.2.1.3 Digestion of gene fragments and vector by restriction enzymes

The 10 - 20 µl of pColdI expression vector and pGEM-T easy vector containing genes of interest were digested in 50 µl reaction with restriction enzymes *Nde*1 (1 µl), *Sal*1-HF (1 µl), 10x Cutsmart buffer (5 µl) and MQ water. The samples were incubated at 37ºC overnight. After that, the samples were kept in a dry block heater at 80ºC for 20 minutes to inactivate the restriction enzymes. Then, the 50 µl sample containing pColdI vector was treated with 1 µl Antarctic phosphatase (AP) in 6 µl 6x AP buffer and 3 µl MQ water to prevent self-ligation of plasmid at 37ºC for 30 min and then the sample was incubated at 80ºC for 2 min to inactivate the Ap. The plasmid DNA were extracted using QIAquick®Gel Extraction Kit (Qiagen) (Appendix E2). For checking successful digestion, the digested pColdI vector sample (5 µl) was loaded into 1% agarose gel and running with TBE (Tris/Borate/EDTA) buffer

(Appendix D2.1). The other two samples containing pGEM-T easy vector with inserts (50 µl) were loaded into 2% agarose gel and running with SB (Sodium Borate) buffer (Appendix D2.2).

5.2.1.4 Ligation of genes of interest into expression vector

After digestion using restriction enzymes and running the gel, the gel bands of inserts (DS44 and DS79) were cut and their DNA was extracted using QIAquick®Gel Extraction Kit (Qiagen). The DNA concentrations were measured using NanoDrop (ND-8000) spectrophotometer. Following, the genes of interest DS44 (2.5 µl) and DS79 (4 µl) were ligated into expression vector pColdI (3 µl) in 10 µl reaction with 0.5 µl T4 ligase, 1 µl 10x ligase buffer and MQ water. The reaction was carried out at room temperature for 3 hours. The recombinant plasmids containing pColdI vector and inserts were transformed into DH5α cells by heat shock method and then spread onto pre-warmed LB agar plates containing Ampicillin (100 µg/ml) to select for the cells that were successfully transformed. The plates were incubated at 37^{o}C overnight. A single colony from streaked plates was picked up and then inoculated in 5ml LB medium containing Ampicillin (100 µg/ml) at 37^{o}C overnight with shaking. The cells were collected by centrifugation at 4000 rpm for 5 minutes. The plasmid DNA was extracted from the cell pellet by using the AccuPrep® Nano-Plus Plasmid DNA Extraction Kit (Bioneer). The plasmids containing the inserts (pColdI/DS44 and pColdI/DS79) were cut again by restriction enzymes *Nde*1 and *Sal*1-HF for screening of successful ligation. The digestion reaction was set up as following:

Component	pColdI/DS44	pColdI/DS79
Plasmid DNA	4 µl	10 µl
10x Cutsmart buffer	1.5 µl	1.5 µl
*Nde*1	0.5 µl	0.5 µl
*Sal*1-HF	0.5 µl	0.5 µl
MQ water	9 µl	3 µl
Total	15 µl	15 µl

The samples were incubated at 37ºC for 2-3 hours and then 5 µl the samples were loaded into 1% agarose gel and running with TBE buffer

5.2.1.5 *E. coli* BL21 transformation and protein expression

The pColdI/DS44 and the pCold/DS79 plasmids (5 µl) were transform into *E. coli* BL21 (DE3) cells (100 µl) by the heat shock method and then spread onto pre-warmed LB agar plates containing Ampicillin (100 µg/ml). The plates were incubated at 37ºC overnight. A single colony from the streak plates was picked up and then inoculated in 5ml LB medium containing ampicillin (100 µg/ml) in 100 ml Erlenmeyer flasks at 37ºC overnight with shaking. The growth of cultures was measured at OD_{600} and then diluted in 5 ml fresh LB/Ampicillin medium to obtain an OD_{600} of 0.2.

The recombinant *Escherichia coli* BL21 (DE3) harbouring the pColdI/DS44 or DS79 alginate lyase plasmid was subcultured for 1–2 hours until the OD_{600} reached 0.5–0.6 in a shaking incubator at 250 rpm and 37°C. The cultures were cooled down to room temperature and induced expression by adding 1mM IPTG after culture has reached OD_{600} 0.5-0.6. On the other hand, to determine the optimal IPTG

concentration for expression, four different concentrations of 0.05 mM, 0.1 mM, 0.5 mM, and 1 mM IPTG were used. After induction, the cultures were incubated at 15^{o}C with shaking at 150 rpm for 24 hours to express the alginate lyase. The cells were harvested by centrifugation at 10,000 rpm at 4^{o}C for 20 minutes. Each sample of cell pellet was dissolved in bacterial lysis buffer (Appendix D5) (40 µl lysis buffer per 1 OD unit) and then 5 µl of the cell lysate were loaded into 4-20% Criterion^{TM} TGX Stain-Free^{TM} Precast Gel (Bio-rad) to check the success of the protein expression.

5.2.2 Western Blot

5.2.2.1 Sample preparation and running the gel

The *E. coli* cell pellet (1g) was dissolved in 30 ml lysis buffer (50 mM Tris-HCl buffer (pH 8.0), 300 mM NaCl and 20 mM imidazole) and was disrupted using high pressure cell disruption equipment (Constant Systems Limited, UK). The cell disruptor was performed at a pressure of 30 kpsi at 4^{o}C. After disruption, the cells were centrifuged at 10,000 rpm for 20 minutes at 4^{o}C. Both the soluble (supernatant) and insoluble (cell pellet) fractions were analysed by a 4-20% Criterion^{TM} TGX Stain-Free^{TM} Precast Gel (Bio-rad) that was described at section 2.6.1. If the gel was to be used for Western Blot, Precision Plus Protein^{TM} Dual Colour Standards (Bio-Rad) was loaded as the molecular weight marker.

5.2.2.2 Transfer

Once the gel run was completed and the image taken, the transfer procedure commenced. Transfer of the protein bands to membrane was performed using a semi-dry method with a Trans-Blot® Turbo^{TM} system (Bio-Rad). The gel and

membrane were assembled into a blotting sandwich as shown in Figure 5.3. The Pre-cut Transfer Pack (Bio-Rad) includes pre-cut Polyvinylidene fluoride (PVDF) membrane and the blotting pads (2 sets). The pre-cut PVDF was briefly soaked in methanol for 1 min with shaking at 50 rpm before it was immersed in 1x transfer buffer (Appendix D3) for a minimum 2-3 minutes. The blotting pads were placed in the transfer buffer and allowed to soak. After that, one pre-soaked blotting pad was set onto the Trans-Blot Turbo Tray, then PVDF membrane was place onto the blotting paper, the gel gently placed on top of the PVDF and the final blotting pad was set on top of the gel. The sandwich was softly rolled to remove any bubbles. The tray lid was placed on top and tightly locked. Proteins were transferred using the standard protocol for a mini gel (Mixed Mw 7min) at 25V and up to 1 A. To check transfer efficiency, both the gel and the membrane were imaged using the Gel Doc EZ^{TM} imager (Bio-Rad).

Figure 5.3: A blotting sandwich for protein transfer from gel to PVDF membrane using semi-dry system.

5.2.2.3 Blocking and primary antibody incubation

After transfer was completed, the membrane was removed from the unit and placed in a tray containing 5% skim milk powder solution in 1x PBS-T (0.1% Tween 20) to block for 1 hr with shaking. After that, the membrane was washed briefly with wash buffer (1xPBS-T). The primary antibody (Anti 6xHis-tag monoclonal mouse lgG2b antibody) was diluted 1: 10.000 with 0.1% skim milk powder solution in 1x PBS-T. The membrane was incubated with the primary antibody overnight at 4^{o}C with shaking at 50 rpm.

5.2.2.4 Secondary antibody incubation and detection

Non-binding primary antibodies were removed by washing 3 times with the wash buffer for 10 min before exposure to secondary antibody (diluted 1:15,000 in 1x PBS-T), which was 1R Dye 680 RD Donkey anti-mouse (LI-COR, Millennium Science, AU) for 1 hour at room temperature with shaking.

The unbound secondary antibody was washed out from the membrane for 10 min/ 3 times using the wash buffer. The membrane was detected by fluorescence using Odyssey® CLx Imaging System (LI-COR, USA)

5.2.3 Partial Purification of Alginate Lyase

The insoluble fraction was dissolved in 3 ml of buffer A (50 mM Tris-HCl buffer (pH 8.0), 300 mM NaCl, 20 mM imidazole and 8 M urea) and stirred at room temperature for 1 hours. The supernatant obtained after centrifugation at 10,000 rpm for 20 minutes at 4^{o}C was passed through a 0.22 µm filter. The supernatant containing 6xHis-tagged alginate lyase was loaded on a Ni-Sepharose 6 fast flow column (GE Healthcare) and equilibrated with buffer A. The unbound proteins were removed by washing the column with buffer A. To refold the bound proteins onto the column, the column was washed with a linear gradient of urea (8 M – 0 M) in buffer A. Finally, the bound proteins were eluted with buffer B (50 mM Tris-HCl buffer (pH 8.0), 300 mM NaCl and 250 mM imidazole). All fractions of each step were analysed by 4-20% Criterion^{TM} TGX Stain-Free^{TM} Precast Gels. The activity and protein concentration of supernatant of the cell lysate, flow through and eluted fraction were measured by DNS and BCA assay as described at section 2.3.2 and 2.3.3.

5.2.4 Molecular modelling of alginate lyase

The three-dimensional structure of DS44 and DS79 alginate lyase were constructed based on the homologues structure of AlyPG alginate lyase from *Corynebacterium* sp. ALY-1 (PDB ID: 1UAI), using Protein Homology/analogy Recognition Engine, version 2.0 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). PyMOL, version 2.3 (http://www.pymol.org) was used to analyse and visualize the modelled structure of these enzymes.

5.3 Results and discussion

5.3.1 Cloning of alginate lyase in *E. coli*

The alginate lyase genes from strain DS44 (*Streptomyces luridiscabiei*) and strain DS79 (*Streptomyces sundarbansensis*) were heterologously expressed in *E. coli* BL21 (DE3) with pColdI expression vector.

First of all, pColdI vector was transformed into DH5α cells, while Gblock gene fragments of DS44 and DS79 alginate lyase were ligated into pGEM-T easy vector and then also transformed into DH5α cells. The DNA plasmids of pColdI vector and pGEM-T easy vector with inserts were extracted and digested by *Nde*1 and *Sal*1-HF restriction enzymes. The digestion results were shown in Figure 5.4. As seen on the gel, the bands of cut vector showed approximately right the size of the pCold (4407 bp) (Figure 5.4 A). On the other hand, in Figure 5.4 B, there were two bands from lane 1 and 2 in which the band on top represented to pGEM-T easy vector (~ 3000 bp) and the other one is alginate lyase gene fragment (~ 827 bp). These results have shown the successful digestion reaction. Therefore, the bands of digested

pColdI vector and DS44, DS79 alginate lyase gene fragments were cut to use in the ligation step.

The genes of interest (DS44 and DS79 alginate lyase genes) were ligated into expression vector pColdI by T4 ligase in ligase buffer. Afterward, the recombinant plasmids containing pColdI vector and inserts were transformed into DH5α cells by the heat shock method. The plasmids containing insert (DS44 and DS79 alginate lyase genes) were cut again by restriction enzymes *Nde*1 and *Sal*1-HF and then run agarose gel electrophoresis to verify insertion of the correct DNA fragments. As shown in Figure 5.5, two bands can be seen clearly at the right size of pColdI vector and the insert DNA fragments. It showed that the alginate lyase genes were successfully ligated in pColdI expression vector. Furthermore, the correct inserted DNA fragments were confirmed by sequencing using the following sequencing primers: forward primer (pCold-F 5'-ACGCCATATCGCCGAAAGG-3'), reverse primer (pCold-R 5'-GGCAGGGATCTTAGATTCTG-3'). The results of sequencing were represented at Figure 5.6 and 5.7 showing the correct sequences of DS44 and DS79 inserted fragments, respectively.

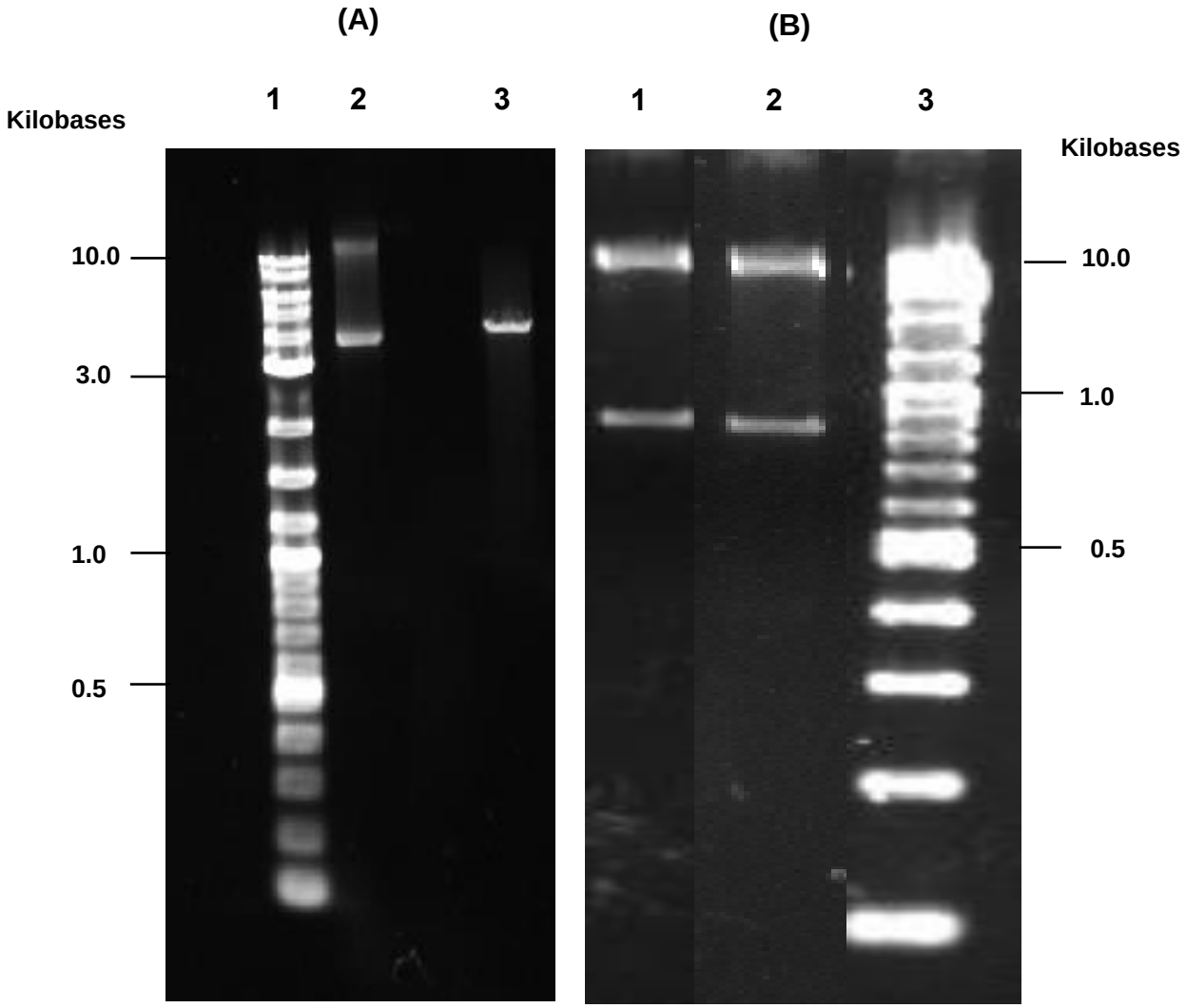


Figure 5.4: The digestion of pColdI vector (A) and pGEM-T/inserts (B) by restriction enzymes.

(A) Lane 1: marker, lane 2: uncut vector, lane 3: cut vector.
(B) Lane 1: DS44 alginate lyase gene fragment, lane 2: DS79 alginate lyase gene fragment, lane 3: marker.

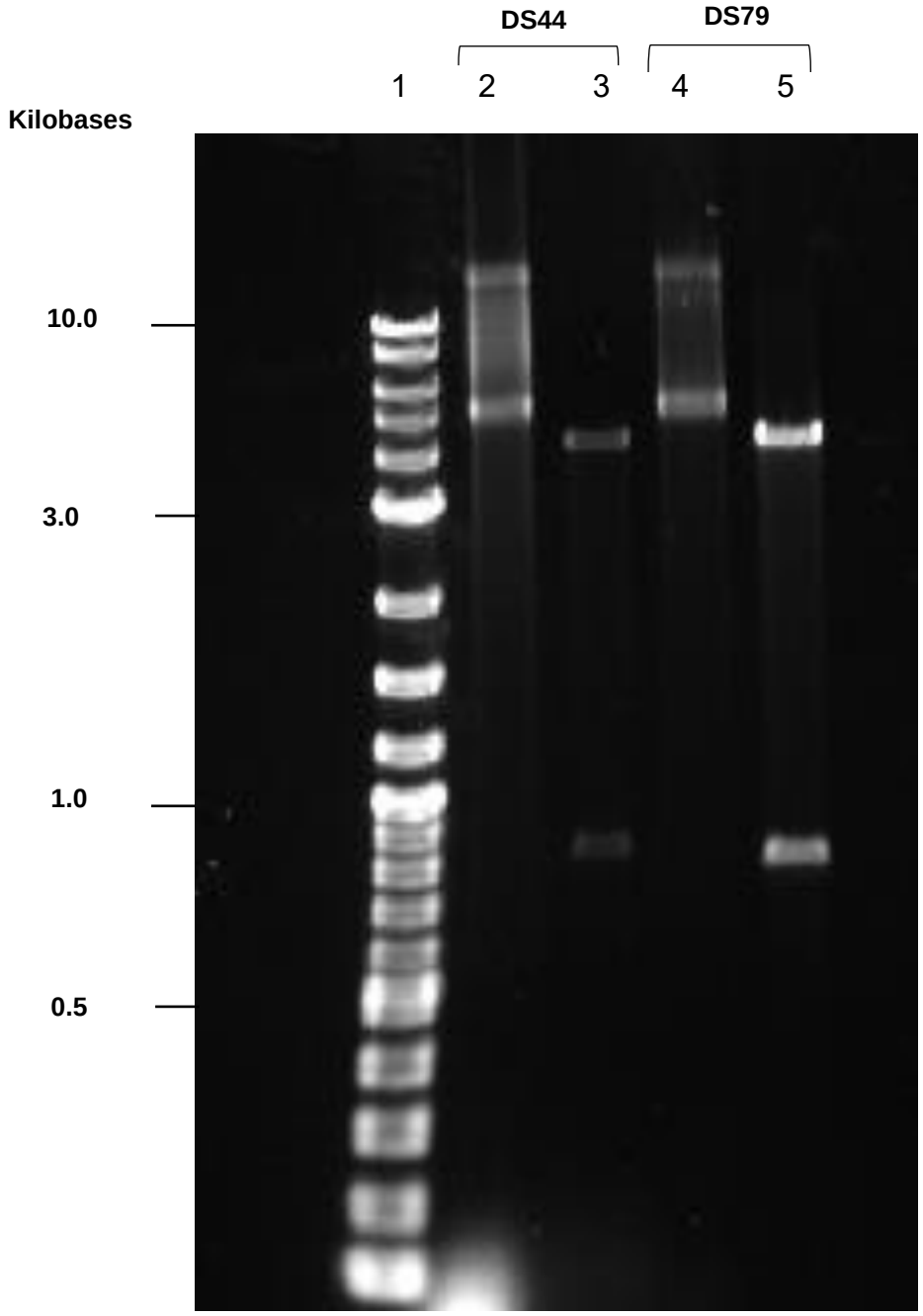
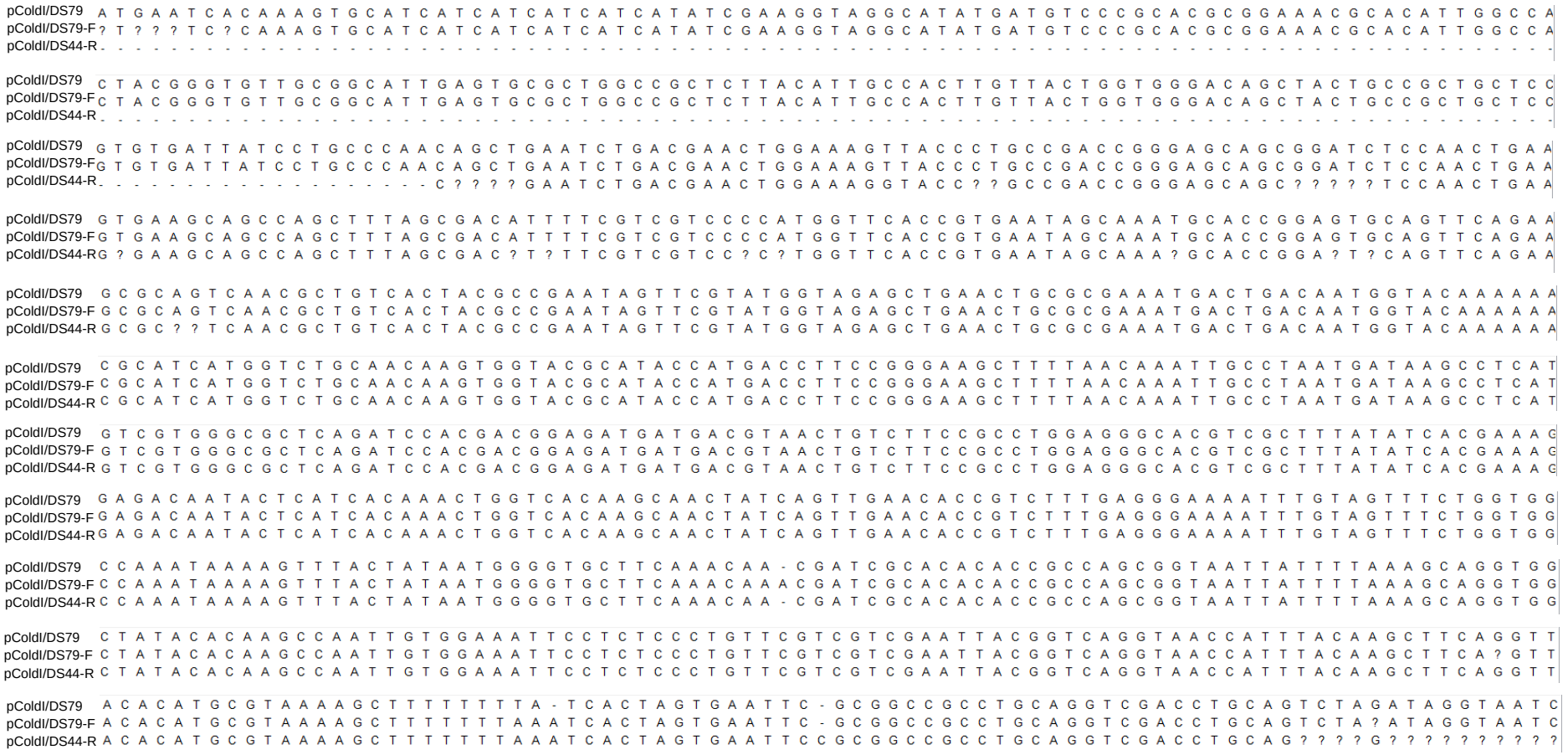


Figure 5.5: The digestion of recombinant plasmids containing strains DS44 and DS79 genes by restriction enzymes. Lane 1: marker, lanes 2 & 4: uncut recombinant plasmids, lanes 3 & 5: cut recombinant plasmids.

Figure 5.6: Sequencing analysis to confirm the correct sequence of DS44 inserted fragment. pCold/DS44-F: sequencing using forward primer, pCold/DS44-R: sequencing using reverse primer, pCold/DS44: the correct DS44 inserted fragment in pColdI vector was checked by ApE edit vector software

Figure 5.7: Sequencing analysis to confirm the correct sequence of DS79 inserted fragment. pCold/DS79-F: sequencing using forward primer, pCold/DS79-R: sequencing using reverse primer, pCold/DS44: the correct DS79 inserted fragment in pColdI vector was checked by ApE edit vector software.



5.3.2 Expression of alginate lyase in *E. coli*

Based on the description of Takara Bio, pColdI is one of four cold-shock expression vectors (pCold DNA I-IV) which provide an increase in *in vivo* protein yields, solubility and purity for recombinant proteins. The development of pCold DNA depended on a *cspA* gene encoding a cold shock protein. pCold DNA contains the *cpsA* promoter and 5' untranslated region (UTR), which becomes highly stable at the low temperature range. This led to the improvement of efficient translation and protein synthesis at low temperature (15^{o}C). Shin *et al.* (2011) also reported that low temperature affected positively on the soluble expression of marine alginate lyase in *E. coli* due to slowing down the translation rate, resulting in an improvement of the proper folding as a soluble form. Therefore, in this study, pColdI expression vector was employed and expression step was performed at low temperature. In detail, the recombinant *E. coli* BL21 (DE3) harbouring the pColdI/DS44 or DS79 alginate lyase plasmid was cultured on a LB medium containing 100 µg/ml ampicillin and incubated at 15^{o}C for 24 hours to express the alginate lyase gene by addition of 1mM IPTG. The cells were harvested and dissolved in bacterial lysis buffer for running SDS-PAGE. As shown in Figure 5.8, recombinant DS44 and DS79 alginate lyases in pColdI vector were successfully expressed in *E. coli* BL21. This was indicated by the obtained protein bands with the sizes of approximately 30.5 KDa, corresponding to the predicted molecular mass of the recombinant DS44 and DS79 alginate lyases.

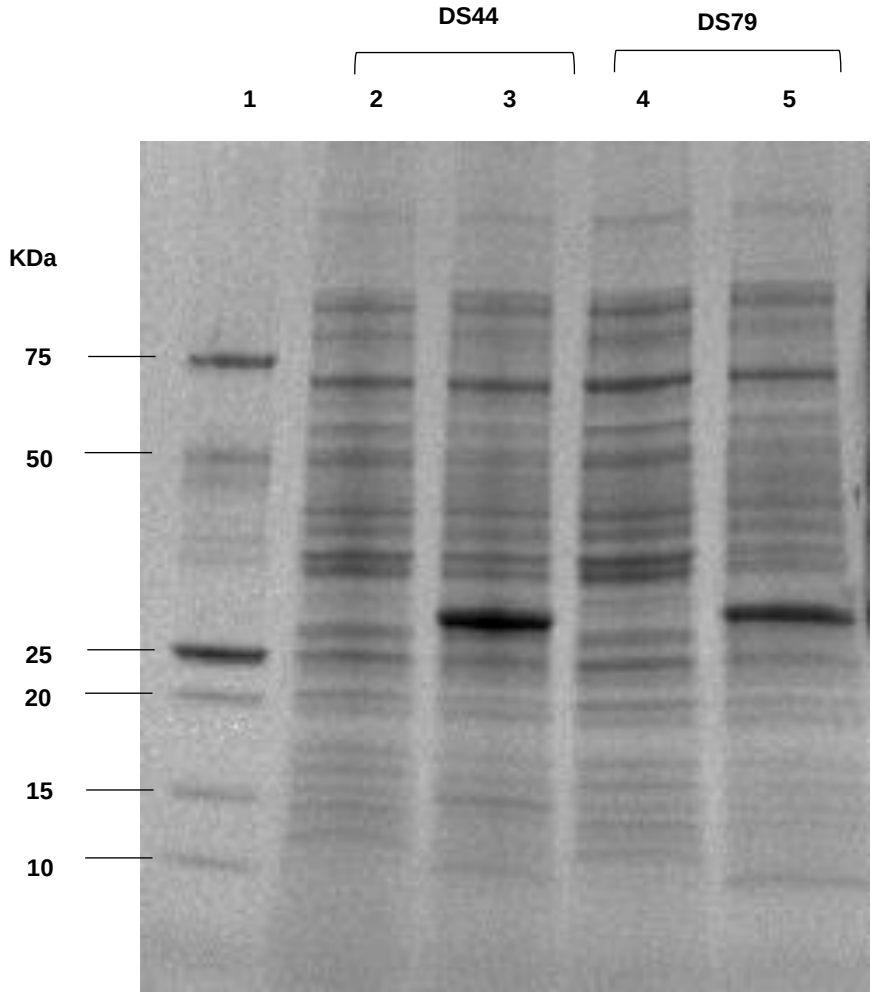


Figure 5.8: Expression of DS44 and DS79 alginate lyases in *E. coli* by induction with IPTG. Lane 1: marker, lane 2 & 4: expressed protein without IPTG, lane 3 & 5: expressed protein with 1mM IPTG.

IPTG is usually utilized as an inducer for expression due to its ability to bind to the lacI repressor and alter its conformation, which prevents the binding between the lac repressor and the lac operator. Therefore, IPTG has been used to induce the expression which forms active proteins (Silaban *et al.*, 2019). The concentration of IPTG is a vital factor which requires investigation due to its influence on overall costs, protein yield, the total biomass productivity, and its effect on the inhibitory of cell growth (Wang *et al.*, 2018). Furthermore, it has also been reported that the level of IPTG concentration affects and influences protein folding processes (Rizkia *et al.*, 2015).

To determine the effect of IPTG concentration on alginate lyase expression, the induction was done by four different concentrations of IPTG (0.05 mM, 0.1 mM, 0.5 mM, and 1 mM). As shown in Figure 5.9, *E. coli* successfully expressed the DS44 and DS79 alginate lyase with the molecular weight of approximately 30.5 kDa using various concentrations of IPTG including 0.1 mM, 0.5 mM, and 1 mM. There were no significant differences between concentration of 0.1 mM, 0.5 mM and 1 mM IPTG in the expression of DS44 protein. Therefore, 0.1 mM IPTG was chosen for DS44 alginate lyase expression because of the cost saving. On the other hand, the results exhibited that the optimum value of IPTG concentration was 1 mM for DS79 alginate lyase.

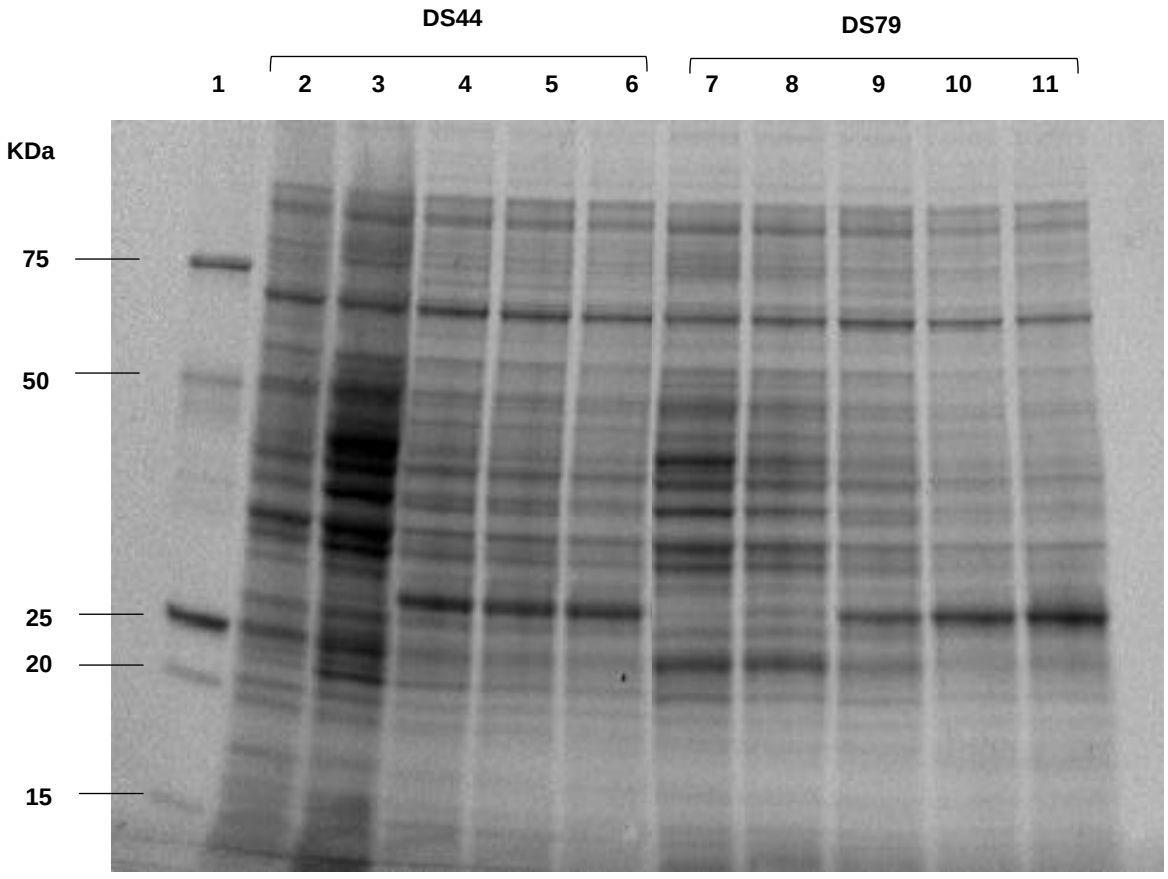


Figure 5.9: Expression of the DS44 and DS79 alginate lyases in *E. coli* by induction with various concentration of IPTG. Lane 1: marker, lane 2 & 7: expressed protein without IPTG, lane 3 & 8: expressed protein with 0.05 mM IPTG, lanes 4 & 9: expressed protein with 0.1 mM IPTG, lane 5 & 10: expressed protein with 0.5 mM IPTG, lanes 6 & 11: expressed protein with 1 mM IPTG.

5.3.3 Partial Purification of recombinant alginate lyase

The recombinant DS44 and DS79 alginate lyase was purified by Ni-Sepharose chromatography. Following expression, harvesting and disruption of the cells (1g of cells were dissolved in 30 ml lysis buffer) were accomplished by using high pressure homogenization technique (Constant systems Limited, UK), which performed at a pressure of 30 kpsi at 4^{o}C. The expression and molecular weight of the alginate lyase was assessed in the supernatant and cell pellet by SDS-PAGE and Western Blot. As can be seen in Figure 5.10, a saturated band at the expected size (around 30.5 KDa) was observed in the cell pellet of each recombinant alginate lyase (lane 3 and 5), while no clear band at this size was visualised in the supernatant (lane 2 and 4). The soluble alginate lyases was too weak to be detected by SDS-PAGE, however, it was evident in immunoblotting analysis by using anti 6xHis-tag antibody (Figure 5.11). The results obtained from SDS-PAGE and Western Blot indicated that alginate lyases were expressed in the soluble fraction as well as an inclusion body, however, most of the target products were produced as insoluble protein (inclusion bodies) rather than as soluble one.

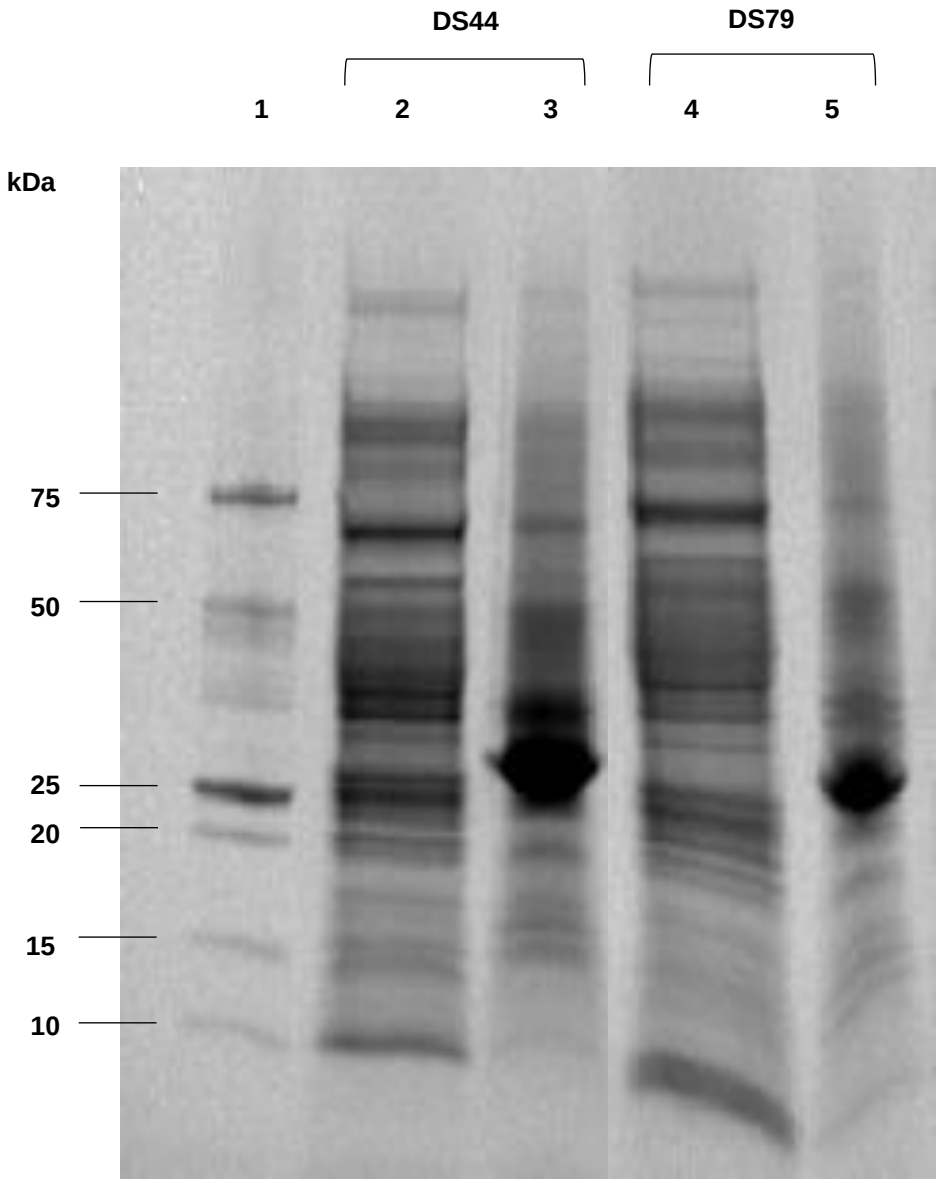


Figure 5.10: SDS-PAGE analysis of the expressed DS44 and DS79 alginate lyases in *E. coli*. Lane 1: marker, lane 2 & 4: soluble proteins; lane 3 & 5: insoluble proteins.

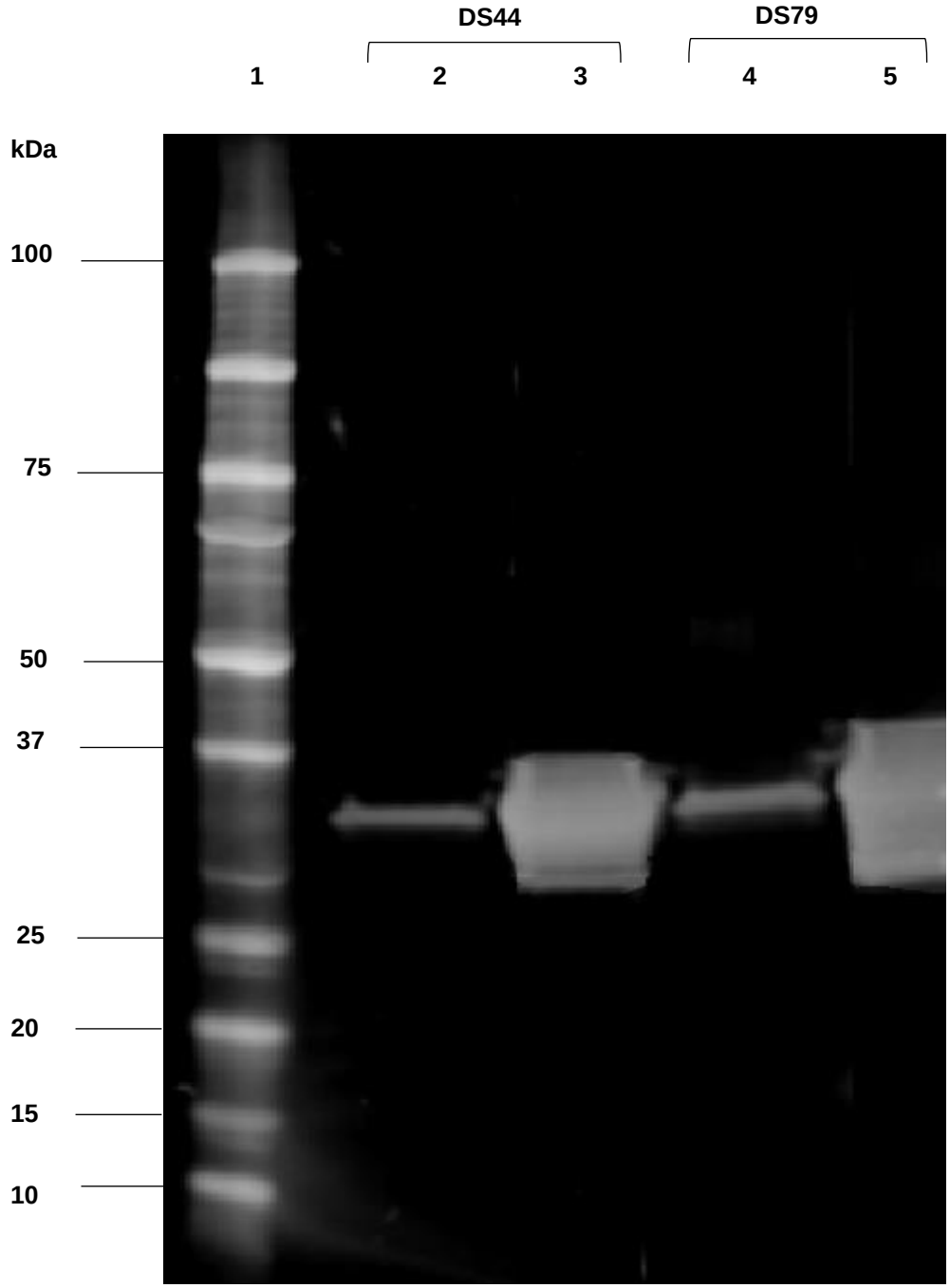
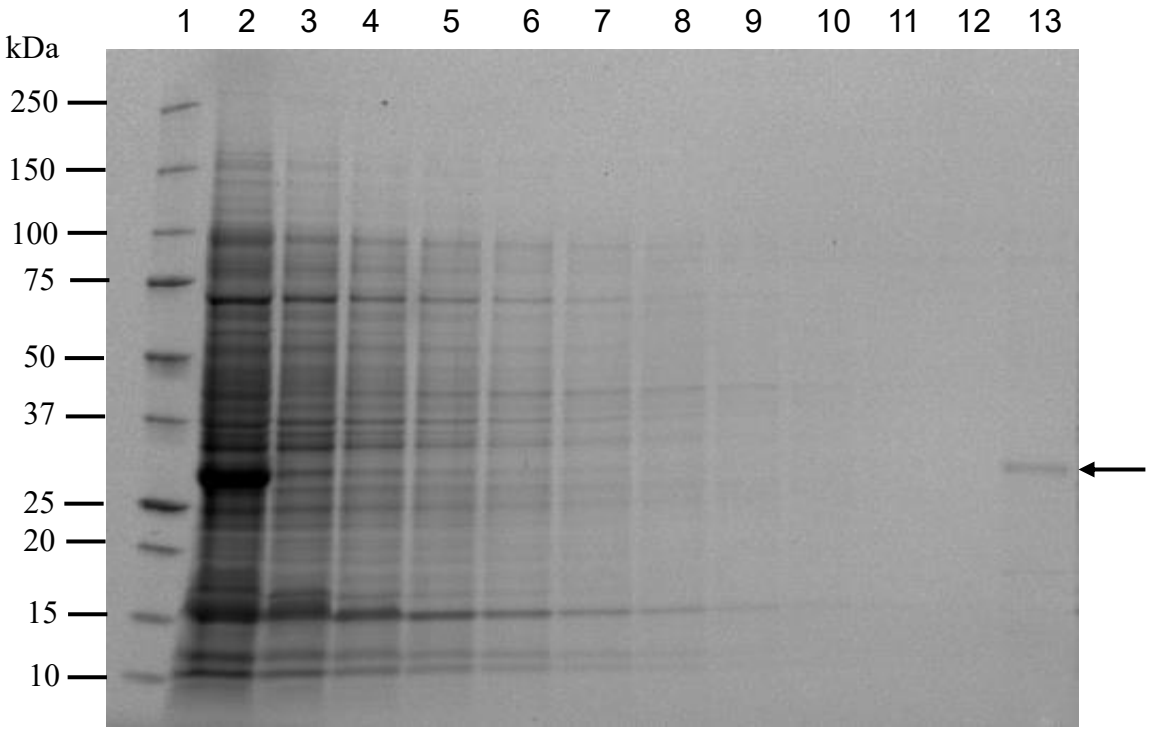
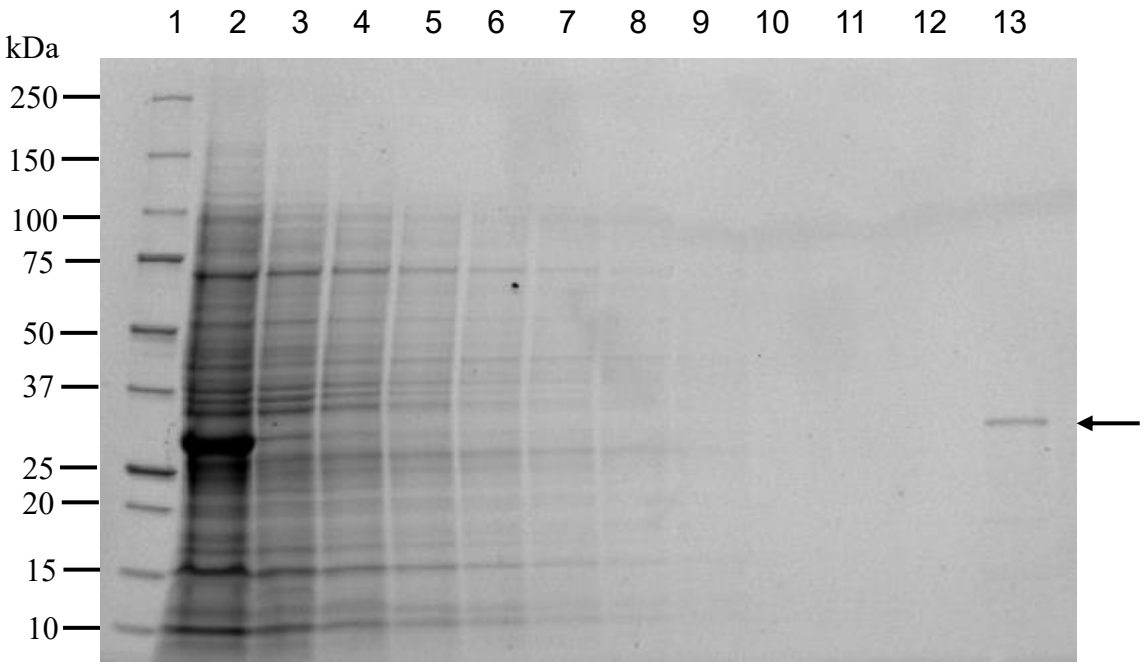


Figure 5.11: Western Blot analysis of the expressed DS44 and DS79 alginate lyases in *E. coli*. Lane 1: marker, lane 2 & 4: soluble proteins, lane 3 & 5: insoluble proteins.



(A)



(B)

Figure 5.12: SDS-PAGE analysis for the purification of the recombinant DS44 (A) and DS79 (B) alginate lyases. Lane 1: marker, lane 2: supernatant of the cell lysate loaded onto Ni^{2+} Sepharose column, lane 3: flow through, lane 4 to 12: wash fractions with a linear gradient of urea (8-0 M), lane 13: eluted fraction.

Table 5.1: Summary of purification of recombinant alginate lyases from strain DS44 (A) and DS79 (B).

Purification Steps	V (ml)	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (Fold)
Supernatant of the cell lysate	3	7.383	18.79	2.546	100	1
Flow through	1	1.214	3.609	2.972	19.2	1.167
Eluted fraction	1	0.075	6.062	80.82	32.3	31.74

(A)

Purification Steps	V (ml)	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (Fold)
Supernatant of the cell lysate	3	6.560	67.56	10.30	100	1
Flow through	1	1.045	7.228	7.875	10.8	0.765
Eluted fraction	1	0.090	7.069	78.54	10.5	7.625

(B)

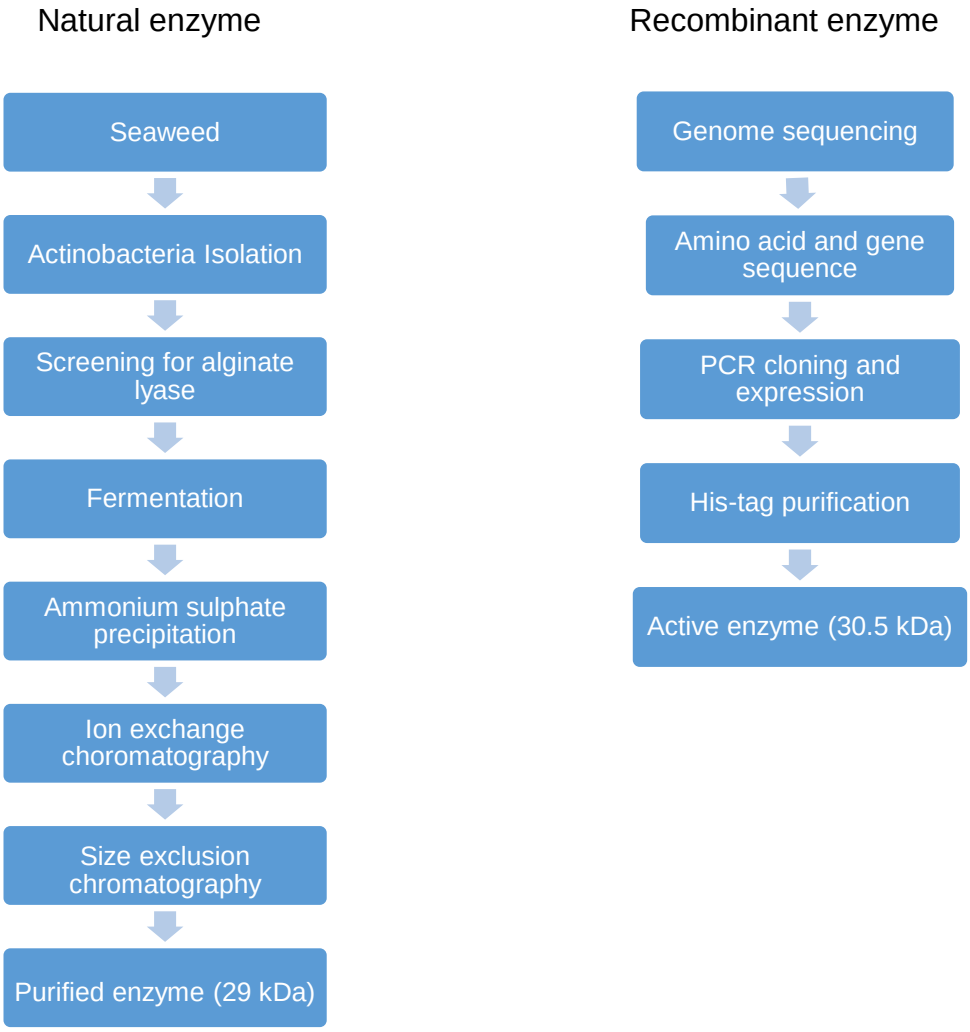
As both the recombinant alginate lyases were expressed as inclusion bodies (insoluble), the proteins were purified under denaturing condition using 8 M urea to dissolve the misfolded protein. The purification of 6xHis-tagged recombinant proteins were accomplished in a Ni^{2+} Sepharose 6 fast flow column. Firstly, the supernatant of the cell lysate (1 ml) was loaded onto a Ni^{2+} Sepharose column. The recombinant alginate lyases were bound to the column while the unbound proteins were washed with buffer A. Refolding of the bound proteins was performed using a linear 8-0 M urea gradient, starting with the wash buffer containing 8 M urea (buffer A), then continue washing with 7 M, 6 M, 5 M, 4 M, 3 M, 2 M, and 1 M urea and finishing without urea. As can be seen in Figure 5.12 A and B, all unbound proteins were completely washed out from the column, the wash fractions of lane 4 to 12 were collected after washing with 8 M to 0 M urea in buffer A. Finally, the refolded recombinant proteins were eluted by using buffer B containing 250 mM imidazole. The eluted fractions were observed as a single band on the gel with the size of approximately 30.5 kDa (lane 13), indicating the successful purification.

Substrate hydrolysis: confirmation of proper refolding of the purified recombinant alginate lyases during purification and biologically active was performed by the DNS assay using sodium alginate as the substrate. 100 µl of His-tagged purified enzyme was incubated with 500 µl of 0.5% sodium alginate in Tris-HCl buffer (pH 8.0) at 37^{o}C for 24 hours. The results shown in Table 5.1 indicated that the specific activity of eluted fractions after purifing by Ni^{2+} Sepharose column were higher than that of supernatant of the cell lyase. The specific activity of DS44 and DS79 alginate lyase was 80.82 U/mg and 78.54 U/mg, respectively, which were higher nearly 30 times and 7 times in comparision with the activity of the cell lyate supernatant (2.5 U/mg for DS44 and 10.3 U/mg for DS79). The yield and the fold of DS44 recombinant

protein were higher than that of DS79 recombinant protein. This suggested that more DS44 recombinant protein had refolded during purification and it was more purity than DS79 protein. Additionally, it can be seen the specific activity of the recombinant alginate lyases were similar to the natural alginate lyase extracted from strains DS44 and DS79 grown in seaweed media (Table 4.1), indicating that the recombinant enzymes retained the similar biological activity.

These results have also shown that the cloning and expression of alginate lyase in *E. coli* was an efficient and useful technique. A similar strategy has previously been employed for purification of the other recombinant alginate lyases including MJ-3 alginate lyase from *Sphingomonas* sp. MJ3, AlyA, AlyB, AlyC, and AlyD from *Vibrio splendius* 12B01, a novel bifunctional alginate lyase FsAlgB from *Flammeovirga* sp. NJ-04, AlyM from *Microbulbifer* sp. Q7 and ALG-5 from *Streptomyces* sp. (Badur *et al.*, 2015; Kim *et al.*, 2009; Park *et al.*, 2012; Yang *et al.*, 2018; Zhu *et al.*, 2019).

The purification strategy of natural and recombinant alginate lyase is shown as follows:



Based on both processes, it is clearly shown that the protocol for recombinant enzyme purification was short, simple and less time consuming. It took just three days to get purified enzyme products compared to two weeks for the previous natural process which extracted enzymes from actinobacteria. Thus, it helped to save a lot of time, energy and materials such as media and chemicals (especially ammonium sulphate for protein concentration). Furthermore, His-tagged proteins could be purified simply using immobilized metal affinity chromatography, while the previous method required multiple purification steps including anion exchange and size exclusion chromatography. Additionally, histidine tags are relatively small and rarely affect the characteristics of the proteins (Gräslund *et al.*, 2008). The use of

immobilised metal ion matrix to facilitate the binding of histidine residues with high affinity in both denaturing and native conditions has proven the His-tag to be a potent tool in terms of protein purification (Bornhorst & Falke, 2000). However, in some downstream applications, it may be desirable to remove the His-tag to prevent interference with downstream functions of the recombinant alginate lyase.

5.3.4 The optimal pH and temperature of recombinant alginate lyase

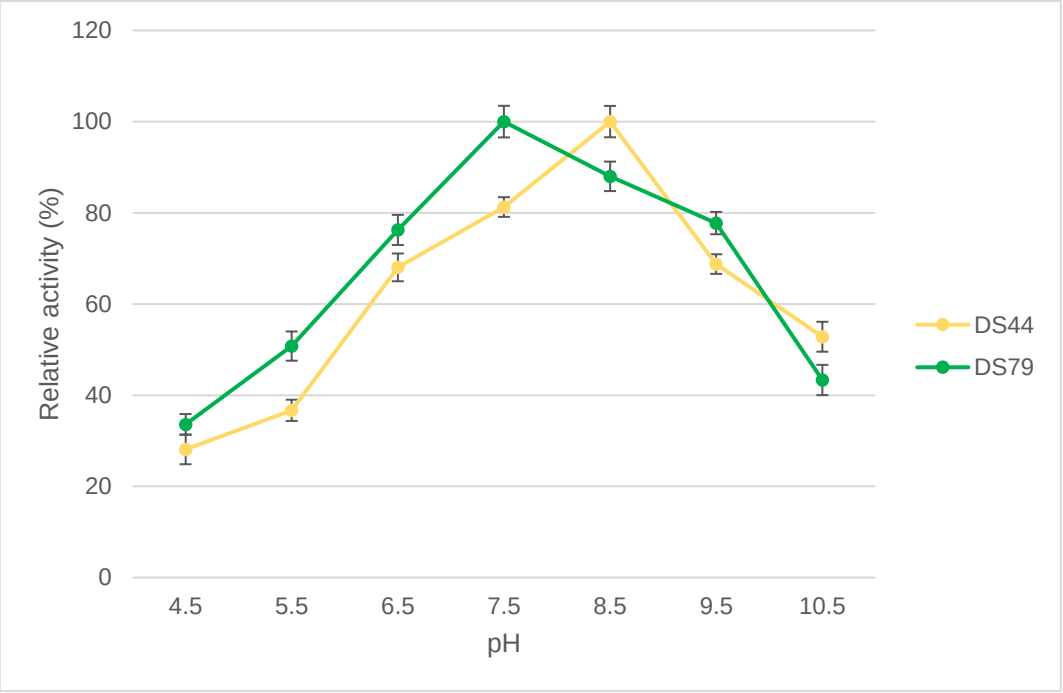


Figure 5.13: Optimal pH of DS44 and DS79 recombinant alginate lyases. The highest activity was set as 100%. Each value represented the mean of triplicates ± standard deviation.

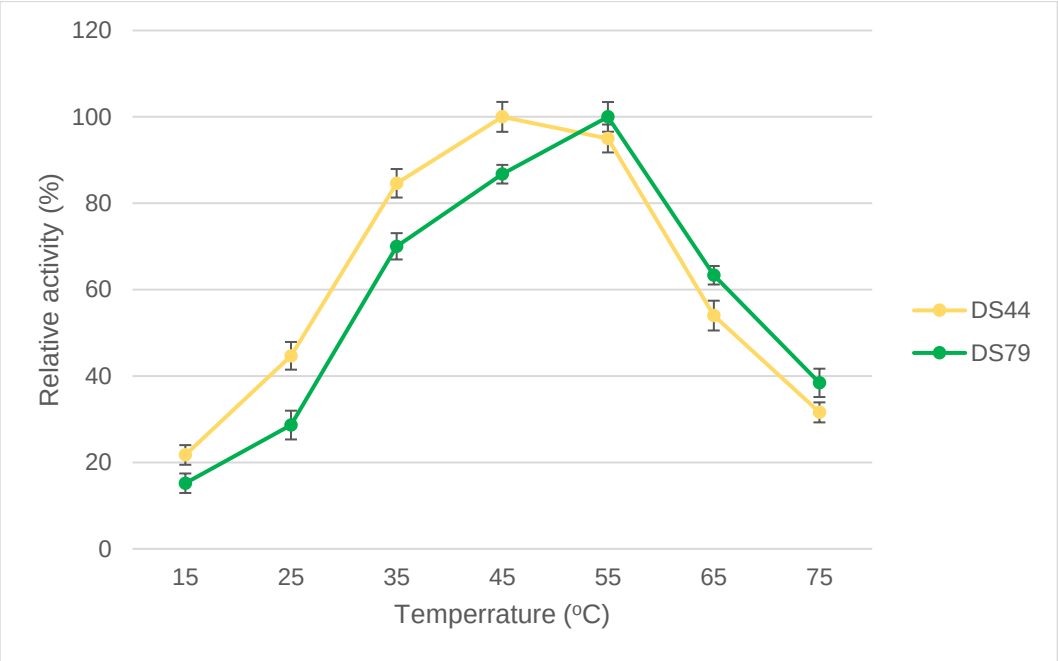
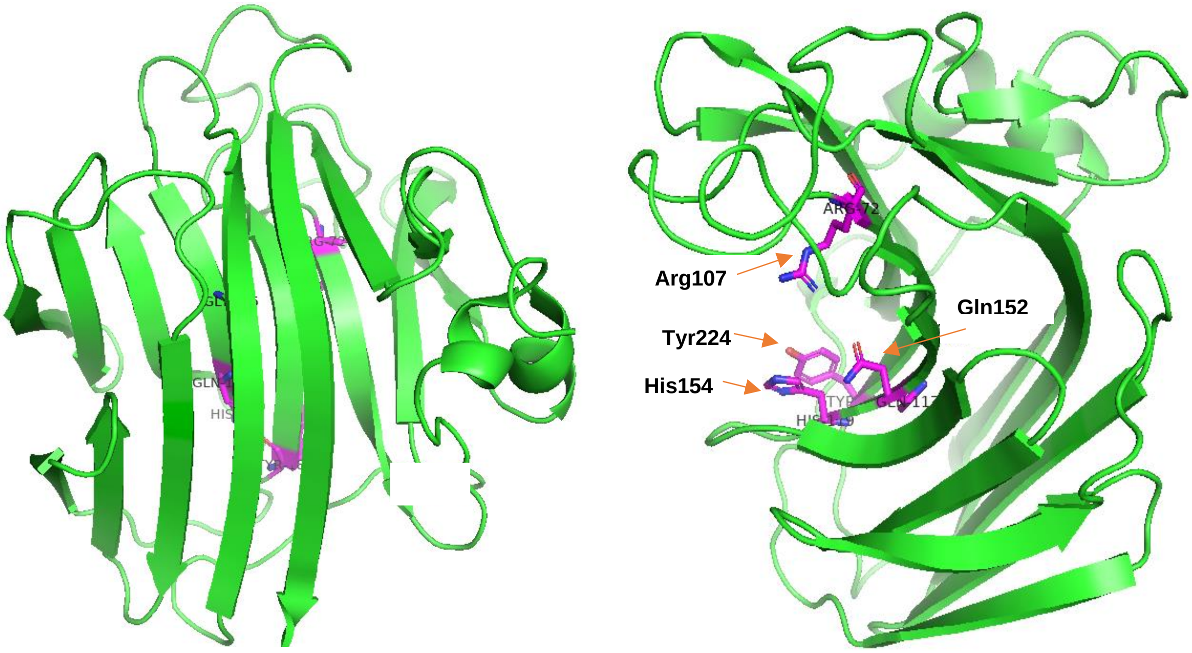


Figure 5.14: Optimal temperature of DS44 and DS79 recombinant alginate lyases. The highest activity was set as 100%. Each value represented the mean of triplicates ± standard deviation.

The optimal pH of DS44 and DS79 recombinant alginate lyse was conducted at 37ºC for 2 hours over a pH range of 4.5 to 10.5 as described in section 2.6.3. The results from Figure 5.13 showed that the maximum activity of DS44 and DS79 recombinant enzymes were obtained at pH 8.5 and 7.5, respectively. DS44 recombinant enzyme keep the similar optimal pH with its natural enzyme (pH 8.5), whereas the optimal pH of DS79 recombinant alginate lyase was 7.5 which is the lower pH compared with the natural one (pH 8.5).

The optimum temperature of DS44 and DS79 recombinant alginate lyases was investigated in 0.02M Tris-HCl buffer (pH 8) with different temperatures from 15ºC to 75ºC for 2 hours as described in section 2.6.3. As shown in Figure 5.14, DS44 and DS79 recombinant enzymes exhibited the highest activities at 45ºC and 55ºC, respectively. There were similar results observed in optimum temperature between recombinant and natural alginate lyases.

5.3.5 Molecular modelling of alginate lyase



(A)

(B)

(C) "Removed due to copyright restriction"

Figure 5.15: The overall structure of DS44 and DS79 alginate lyases with active sites (A), front (left) and side (right) views. Sequence alignment of DS44 and DS79 alginate lyase and a template - AlyPG alginate lyase from *Corynebacterium* sp. ALY-1 (B). The mechanism for alginate lyase reaction (C) - This figure has been reproduced from Badur *et al.,* (2015).

Based on the homologues structure of the AlyPG alginate lyase from *Corynebacterium* sp. ALY-1 (PDB ID: 1UAI), the three-dimensional model of the DS44 and DS79 alginate lyase were constructed using pyMOL (version 2.3) with similarity of 83% and 82%, respectively. The two enzymes have a similar structure, 224 residues (86% of the sequence) have been modelled with 100% confidence by a highest scoring template. The predicted structure of the DS44 and DS79 alginate lyases is shown in Figure 5.15 A, it is a jelly roll β sandwich type protein with two anti-parallel α sheets. The five β strands form the outer convex sheet and the seven β strands which constitute a groove harbouring the catalytic active site form the inner concave sheet (Hu *et al.*, 2019; Zhu *et al.,* 2018, Zhu *et al.,* 2019). According to the multiple sequence alignment (Figure 5.15 B), the highly conserved residues were R107, Q152, H154, and Y224 reported to play a vital role in catalytic activity and involve in the interaction between enzyme and substrates. The possible catalytic mechanism has been proposed that Arg107 (R107) interacts with carboxyl groups of alginate, and His154 (H154) works as a base for the abstraction of proton in a lyase reaction. Gln152 (Q152) and Tyr224 (Y224) interact near the reaction site to maintain the correct enzymatic reaction (Figure 5.15 C) (Osawa *et al.*, 2005).

5.4 Conclusion

In this chapter, the alginate lyase genes from strains of DS44 and DS79 were successfully cloned in pColdI expression vector. The optimal IPTG concentration for induction at 15^{o}C in the *E. coli* BL21 (DE3) expression system were 0.1 M and 1 M against DS44 and DS79 alginate lyase, respectively. The His-tagged proteins were purified effectively by using Ni^{2+} Sepharose affinity chromatography and refolded efficiently with a linear gradient of urea (8-0 M). Biocatalytic function analysis indicated that the activity of recombinant alginate lyase could be recovered in terms of the degradation of alginate. DS44 and DS79 recombinant enzymes have the optimal pH of 8.5 and 7.5; and the optimal temperature of 45^{o}C and 55^{o}C, respectively. Therefore, cloning and expression of recombinant protein is a suitable technique that can be applied for the large scale production of commercial alginate lyase for industrial processes.

CHAPTER 6: APPLICATIONS OF ALGINATE LYASE

6.1 Introduction

In recent years, alginates and their oligosaccharides have been used broadly in the food, pharmaceutical, biomedical and biotechnological industries due to their potent and effective properties as a biodegradable, biocompatible and mucoadhesive polymers (Iskenderoglu *et al.*, 2013). As a polysaccharide, the molecular weight of alginate is one of the determining factors that affects its physical properties, such as viscosity. The commercial sodium alginates often have a molecular weight range of 32,000 and 400,000 g/mol (Lee & Mooney, 2012). However, an alginate solution formed from high molecular weight polymer becomes greatly viscous, which is often undesirable in food, pharmaceutical, biomedical and biotechnological processing (LeRoux *et al.*, 1999).

Low molecular weight alginate has been utilised previously in numerous research projects resulting in the production of biomaterials to be applied in various biomedical applications. In the study by Iskenderoglu *et al.* (2013), alginate was used to develop low molecular weight heparin-alginate beads for oral administration. The alginate beads containing low molecular weight heparin which consisted of a formulation of a 1:2 ratio (heparin:alginate), exhibited an effectiveness as an anticoagulant, following oral delivery to rabbits. Low molecular weight alginate showed stronger antioxidant activity than alginate polymer and it could be useful for industrial and biomedical applications (Kelishomi *et al.*, 2016; Sen, 2011).

The antimicrobial activity of alginate with a particular structure had been reported widely (Friedman *et al.*, 2013; Kumar *et al.*, 2019; Oussalah *et al.*, 2006; Park *et al.*, 2016; Patel *et al.*, 2019; Salem *et al.*, 2019; Sen *et al.*, 2017; Shankar & Rhim, 2018; Tang *et al.*, 2018). The cellulose nanofibrils (CNF) which incorporated the low-

molecular-weight alginate oligosaccharide OligoG CF-5/20 has been investigated to evaluate their structural and antimicrobial properties (Jack *et al.*, 2019). The results obtained indicated that as aerogels or films, the OligoG-CNF formulations inhibited pyocyanin production, and additionally they have the ability to inhibit any bacterial growth and related biofilm development. Therefore, OligoG and CNF bionanocomposites were presented as potential candidates to be utilised in biomedical applications in terms of prevention of infection or biofilm growth.

Another interesting study demonstrated that alginate lyase had been developed as an anti-biofilm agent (Li *et al.*, 2019). It was used to degrade alginate which was the main component exhibiting the properties that cause surface adhesion and stabilisation of the biofilm of *Pseudomonas aeruginosa*. In this study, the low molecular weight chitosan and alginate lyase Aly08 from marine bacteria *Vibrio* sp. SY01 were used for chitosan nanoparticles synthesis (AL-LMW-CS-NPs). As a result, the high efficiency of the immobilized AL-LMW-CS-NPs was observed in regard to inhibition of biofilm formation and interruption of the established mature biofilm of *P. aeruginosa*. Moreover, the antibiotic sensitivity of *P. aeruginosa* had increased dramatically due to the disruption of the biofilm.

Calcium alginate beads are increasingly used as a biomaterial in a wide range of applications, partially in the encapsulation of drugs as they can offer regulated and controlled delivery, which extends further the potential uses for alginate and alginate lyase (Acarturk & Takka, 1999; Wong *et al.*, 2000).

From all of the above studies, it can be seen that demand for the oligoalginates resulting from efficient production techniques increases daily. Thus, the alginate

lyase with high endo-enzyme activity targeting the degradation the alginate will become essential element.

There are two objectives covered in this Chapter:

1. To screen actinobacteria derived from a range of sources for their alginate lyase activity to better determine the best source of actinobacteria for further evaluation of enzymes.

2. To determine the breakdown products formed by the application of the enzymes to low viscosity sodium alginate. The molecular weight analysis using HPLC was also carried out to test the ability of recombinant DS44 and DS79 alginate lyases in terms of degrading the low viscosity sodium alginate to the ultra-low molecular weight alginate.

6.2 Materials and methods

6.2.1 Screening alginate lyase genes from actinobacteria from different sources

The materials used in this study include actinobacteria - 80 strains isolated from seaweed, 100 strains isolated from marine sponge, 100 strains isolated as endophytes from plants, and 100 strains isolated from soil.

The seaweed samples were collected at Rivoli Bay, Beachport, South Australia and processed to isolate actinobacteria as described in section 2.2.2 and 2.2.3.

The sponge samples were collected at Glenelg blocks and Rapid Bay Jetty of South Australia by scuba diving. The small pieces (approximately 1 cm^3) of dried sponges were homogenized using a pestle and mortar and autoclaved seawater. The dilution

was produced in a series ranging from 10^{-1} to 10^{-6} and 100 µl of three dilutions of 10^{-4}, 10^{-5}, and 10^{-6} were inoculated onto seven isolation media including Starch yeast peptone agar (SYP), Asparagine peptone agar (ASP), Natural seawater agar (SWA), Humic acid vitamin agar (HV), ½ strength Nutrient agar (NA), Marine agar (MA) and ½ strength Tryptone soya agar (TSA). The plates were then incubated at 15ºC and 27ºC for 16 weeks (Anteneh, 2019).

The plant samples are from legumes such as chickpea which were grown from the seed of chickpea Kabuli genesis 090. There were 100 endophytic actinobacterial strains were isolated from chickpea roots and nodules by Vo (2018) using Humic acid Vitamin B Agar (HVA) and VL70 with Amino acid medium and incubated at 27ºC for 2 weeks.

Another set of 100 strains was obtained from soil samples collected from arid regions of Central Australia and isolated by Evans (2001). 1g of each soil sample was inoculated into sterile saline and homogenised by sonication. The suspension was diluted to the dilution of 10^{-4}, and then 200 µl of the final dilution of each sample was plated onto four media in agar plates including International Streptomyces Project (ISP4), soil extract agar (SE), colloidal chitin (CC), wool wax agar and potato dextrose agar (PDA), containing 50 µg of the antifungal cycloheximide. The plates were incubated at 27ºC for 4 to 6 weeks and the isolates grown on HPDA and stored in sterile 20% (v/v) glycerol at -80ºC.

All the strains were subcultured on HPDA media at 27ºC for at least 7 days to form the spores. Their DNA were extracted by using the CTAB and 100 micron zirconium bead that was described in section 2.8.1. The alginate lyase genes were amplified by running PCR using primers which were designed for encoding alginate lyase

genes, including forward primer: H1F (5'-GCGTGGACATCAAGGAGAAC-3') and reverse primer: H6R (5'-AYCGGGMWAKGTGCGAATS-3'). Finally, gel electrophoresis was used to obtain the bands of amplified alginate lyase genes.

6.2.2 Plate assay for screening alginate lyase

The alginate lyases detected from actinobacteria from different sources were confirmed by using a plate assay with cetylpyridinium chloride (CPC) (Gacesa & Wusteman, 1990; Kim *et al.*, 2013). Firstly, the colonies of each strain were replicated onto 0.5% alginate agar plates. The plates were incubated at 27^{o}C for four days. After four day incubation, the growing cells were removed and then the agar plates were flooded with 10% (w/v) CPC to form an insoluble precipitate when CPC reacted with sodium alginate (Sawant *et al.*, 2015). Therefore, clear haloes were obtained if any actinobacteria could produce alginate lyase which degraded the alginate to oligosaccharides. After flooding with CPC, the plates were kept for 30 minutes at 37^{o}C and then washed three time with MQ water. The plates had visual clear zones were photographed on a black background and the outer diameters of the cleared zones were measured. Additionally, a bigger halo corresponds to a higher degradation activity.

6.2.3 Degradation of low viscosity sodium alginate

The low viscosity sodium alginate was provided by GGOG, QINGDAO Gather Great Ocean Algae Industry Group Co., LTD. It is their low viscosity food grade sodium alginate products.

To test the ability of DS44 and DS79 recombinant alginate lyases in terms of degrading the low viscosity sodium alginate to produce the low molecular weight

degradation products, an HPLC (Shimazu, USA) with a GPC/ SEC column was used and its operation process was described in section 4.2.2.6. The samples were prepared by mixing 100 µl each purified enzyme (75 – 90 µg/ml) and 500 µl the low viscosity sodium alginate at a concentration of 0.5% (w/v) and 1% (w/v) in 0.02 M Tris-HCl buffer (pH 8.0). These samples were incubated at 37^{o}C for 24 hours. 100 µl of each sample was taken at 0h and 24h incubation and diluted two time with 0.1 M sodium nitrate. The samples were centrifuged at maximum speed (12,000 rpm) for 5 minutes and then 100 µl of each sample was placed into a HPLC vials. 50 µl of each sample was injected and run through the column with a flow rate of 1.0 ml/min. The peaks were detected by refractive index (RI) detector.

6.3 Results and discussions

6.3.1 Screening alginate lyases genes

The alginate lyase genes of actinobacteria isolated from various sources including seaweed, marine sponge, plant and soil were amplified by PCR. The PCR results are shown on the agarose gels at Figure 6.1.

The number of actinobacteria from different sources having alginate lyase genes is summarised in Figure 6.2. Seaweed was the best source for the discovery of the alginate lyase producers in comparison with the other sources. Of the actinobacteria isolated from seaweed, approximately 36% possessed alginate degrading enzymes. This was approximately five to eighteen times higher than that of actinobacteria from marine sponge (7%) and plants (2%) respectively. There was only one strain found in soil having alginate lyase out of 100 experimental strains tested. These results indicated that marine actinobacteria isolated from marine

sources such as seaweed or to a lesser extent, marine sponge, could be a promising potential source in terms of polysaccharide-degrading enzymes.

To confirm the PCR assay correlates with the production of functional alginate lyases from actinobacteria, the plate enzyme assays were conducted with the 120 strains which exhibited positive results (40 strains) and negative results (80 strains). After four days incubation at 27^{o}C, the alginate agar plates containing the spores of strains were flooded with 10% (w/v) CPC. The clear zones were formed on the alginate agar medium as shown at Figure 6.3. Additionally, the outer diameter of transparent rings were recorded in Table 6.1. It can be seen, most positive strains showed the transparent halos in which the largest ones belong to strain DS44 and DS79 (3.3 cm and 3.2 cm). Five strains DS6, DS19, DS48, DS73 from decomposing seaweed and YA32-2 from marine sponge that showed a PCR product indicating the presence of the alginate lyase gene showed no degradation activity toward alginate on the agar plates. The possible explanation for these strains having alginate lyase genes by PCR test but no observation in the halo zone on alginate agar plates is that they might grow very slowly on the 0.5% alginate agar medium, which led to very little or no enzyme production and no clear zone obtained after the short incubation time (just 4 days). The alginate lyase gene of a ‘random’ positive strain DS6 was confirmed to be similar to strain DS79 by sequencing of its PCR product (Figure 6.4). On the other hand, there were no transparent halos on the agar plates containing the 80 strains which had negative results in PCR.

To sum up, the technique using primers to detect genes encoding alginate lyase for screening for its presence in the DNA of actinobacteria is more accurate than the plate assay and simpler to identify the alginate lyase gene sequences by way of

sequencing the amplified genes without running mass spectrometry. This would open a new opportunity to discover more novel alginate lyase from a range of natural sources. Based on this, the new structures and multiple bioactive functions of alginate oligosaccharides may be explored.

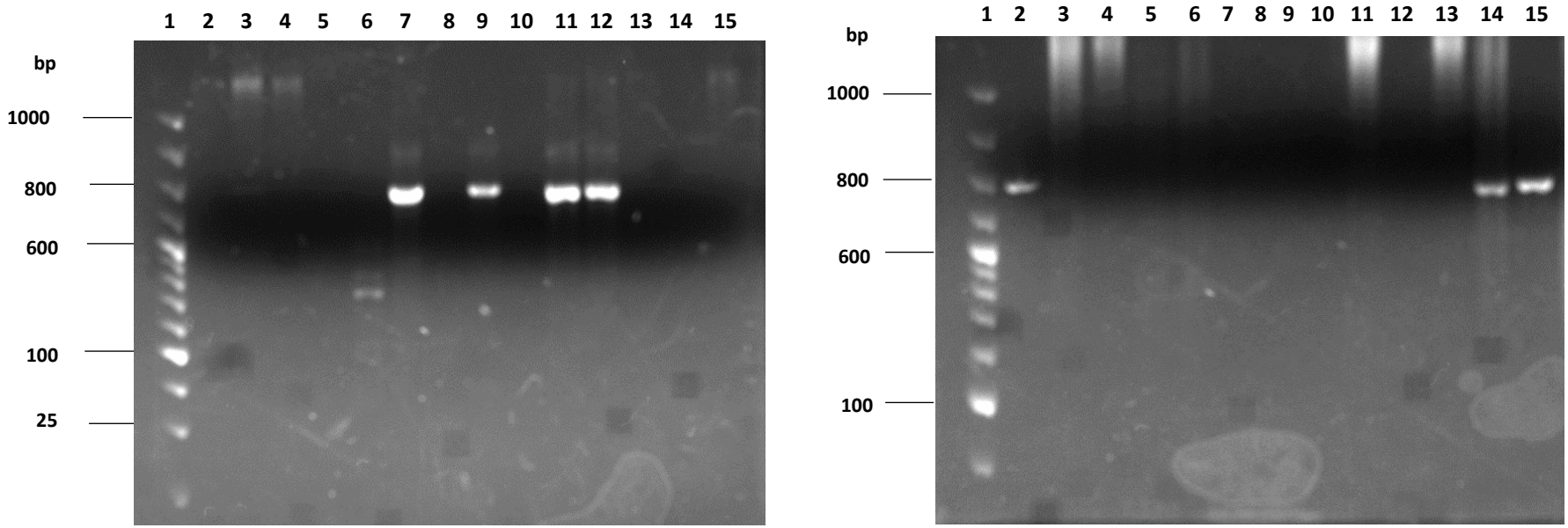


Figure 6.1: Agarose gel electrophoresis for checking the amplification products of alginate lyase genes from actinobacteria isolated from seaweed (A), marine sponge (B), plants (C) and soil (D). Lane 1: 1000 bp DNA ladder, lane 2 – 16: PCR products.

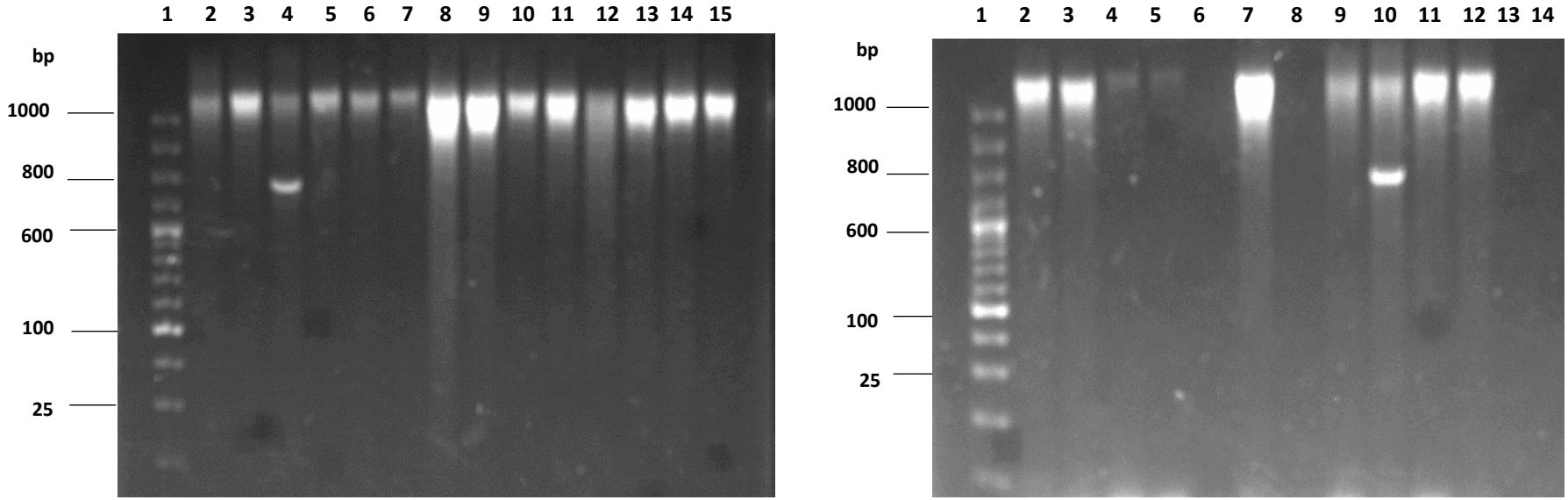


Figure 6.1: Agarose gel electrophoresis for checking the amplification products of alginate lyase genes from actinobacteria isolated from seaweed (A), marine sponge (B), plants (C) and soil (D). Lane 1: 1000 bp DNA ladder, lane 2 – 16: PCR products.

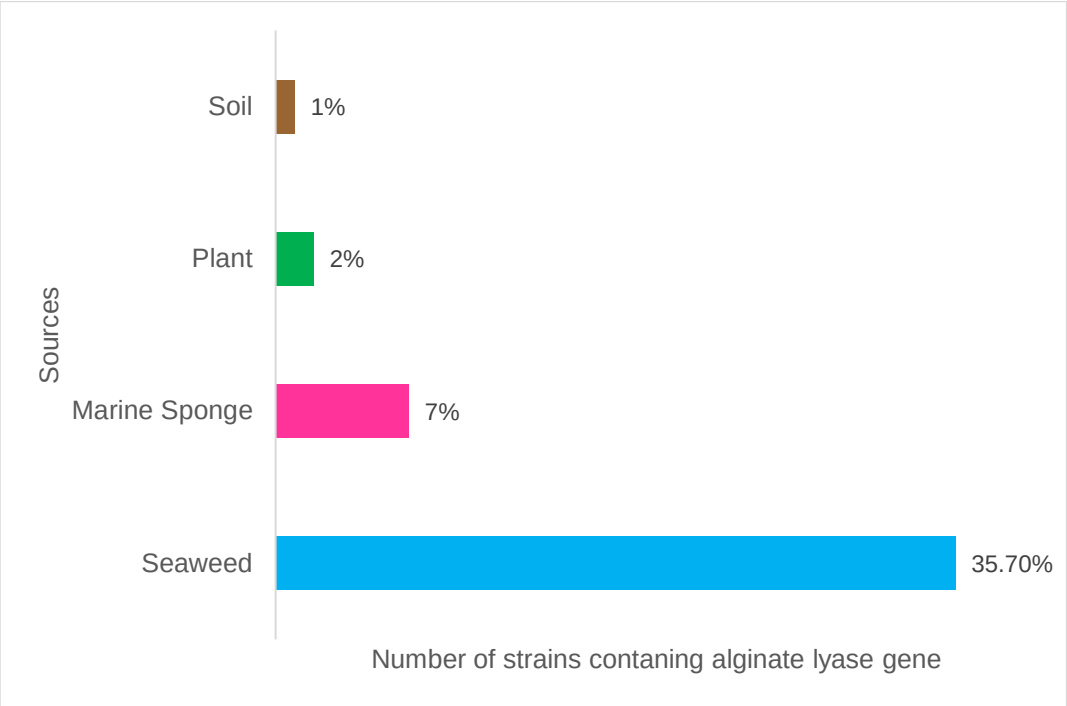


Figure 6.2: Percentage of actinobacteria isolated from different sources of seaweed, marine sponge, plants and soil, containing alginate lyase genes.

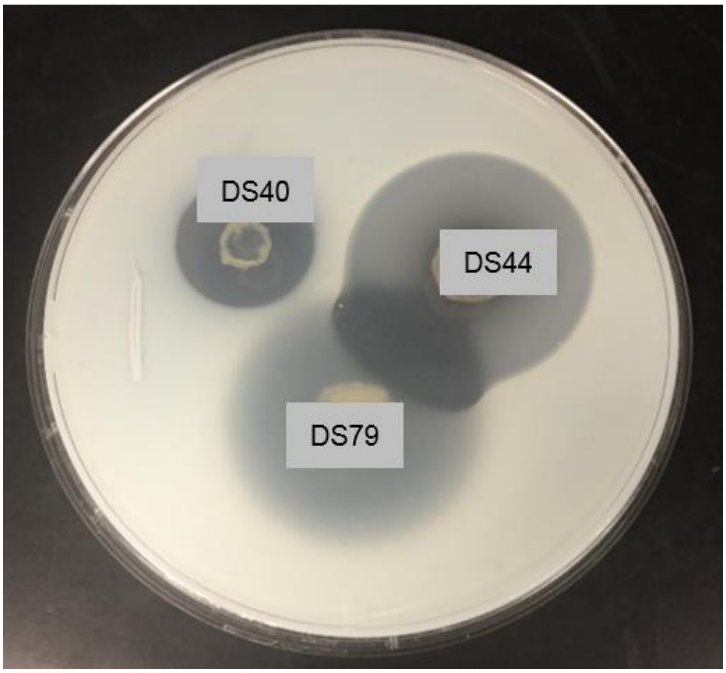


Figure 6.3: Plate assay for the identification of alginate lyase on 0.5% sodium alginate agar plate.

Table 6.1: Summary of plate assay.

Strain ID	Diameter of halos (cm)	Strain ID	Diameter of halos (cm)
Seaweed			
DS6	-	DS59	2.6
DS13	1.5	DS60	1.6
DS17	2.5	DS64	2.5
DS19	-	DS65	2.8
DS20	2.7	DS66	2.5
DS21	2.4	DS68	2.7
DS23	1.9	DS69	0.5
DS24	2.1	DS70	3.0
DS25	1.2	DS71	1.7
DS27	2.3	DS72	2.6
DS31	1.8	DS73	-
DS40	2.9	DS76	2.2
DS44	3.2	DS78	1.7
DS48	-	DS79	3.3
DS55	2.1	DS80	3.0
Marine sponge			
YA7	2.8	YA32-1	2.9
YA7-2	1.7	YA32-2	-
YA8	2.1	Rb182	1.5
YA8-2	2.4		
Plants			
CP28	2.3	CP252	1.8
Soil			
HCA1299	2.0		

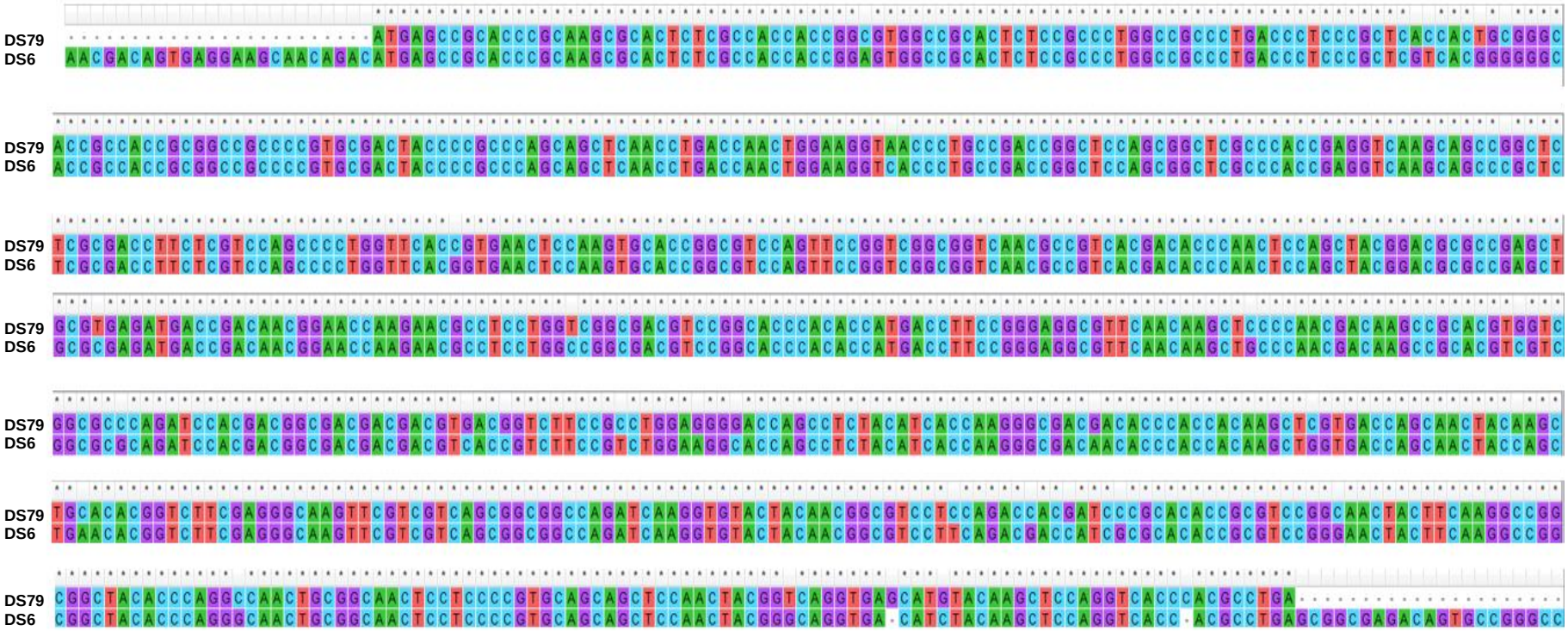


Figure 6.4: Sequencing analysis to confirm alginate lyase gene of strain DS6. The alginate lyse gene sequence of strain DS6 and DS79 were used for DNA alignment by MEGA7 software.

6.3.2 Degradation products of low viscosity sodium alginate

The ability of recombinant alginate lyases in terms of degrading the low viscosity sodium alginate (LVA) to produce the low molecular weight degradation products were realised. The molecular weights of LVA were approximately 552 kDa, 305 kDa and 0.7 kDa, made up of 46.3%, 39.4% and 14.3% respectively. DS44 alginate lyase started to degrade the substrate (0.5% LVA) to oligosacharides after 1 hour and their molecular mass had decreased dramatically after 24 hours incubation at 37^{o}C, accounting for 8.4 kDa (30.4%), 5.6 kDa (30.6%), 3.0 kDa (6%) and 0.7 kDa (33%) (Table 6.2 and Figure 6.5). In comparision with 1% LVA, the main degradation products at 24 hours had a molecular mass of 26 kDa (33.9%) and 9.5 kDa (43.7%) (Table 6.2 and Figure 6.6). These results inferred that 0.5% LVA and the ratio 1:5 (v/v) of the purified enzyme and LVA were appropriate for a degradation reaction, which led to a high production of ultra low molecular weight of alginate oligosacharides.

Similarity, DS79 recombinant alginate lyase also worked effectively in 0.5% LVA and could degrade this substrate into oligosacharides that had approximately a 70 times lower molecular weight after 24 hours incubation (Table 6.3, Figure 6.7 and 6.8). It is interesting that the reaction happened directly at the commencement of incubation, the enzyme could degrade LVA from molecular mass of 552 kDa (46.3%) and 305 kDa (39.4) into 94.6 kDa (24%), 17.8 kDa (27.5%) and 12.6 kDa (24%). This demonstrated that DS79 alginate lyase represented very strong activity on LVA substrate.

It has been reported that the viscosity is mainly related to the average molecular weight (Storz *et al.*, 2010). A high molecular weight alginate can cause an increase

of its viscosity, making it harder to dissolve at high concentration. The low molecular weight alginate will typically dissolve easier in water and has stronger biological activity, therefore, it has a number of advantages in medical and healthy food applications. The results of this experiment indicated that two recombinant enzymes worked very well to further reduce the molecular weight of low viscosity alginate to very low. It surely has the potential to produce ultra-low viscosity alginate for specific applications.

Table 6.2: The change of molecular weight of the low viscosity alginate of 0.5% and 1% after incubation with DS44 alginate lyase at 1h and 24h.

	Peak 1		Peak 2		Peak 3		Peak 4	
	%	MW (kDa)	%	MW (kDa)	%	MW (kDa)	%	MW (kDa)
0.5% the low viscosity alginate								
Control	46.3	552.0	39.4	305.0	14.3	0.7		
1h	21.6	281.5	57.5	151.3	20.9	0.7		
24h	30.4	8.4	30.6	5.6	6.0	3.0	33.0	0.7
1% the low viscosity alginate								
Control	48.7	475.7	43.2	275.8	8.1	0.7		
1h	27.2	281.5	55.3	190.1	17.5	0.7		
24h	33.9	26.0	43.7	9.5	2.8	3.1	19.6	0.7

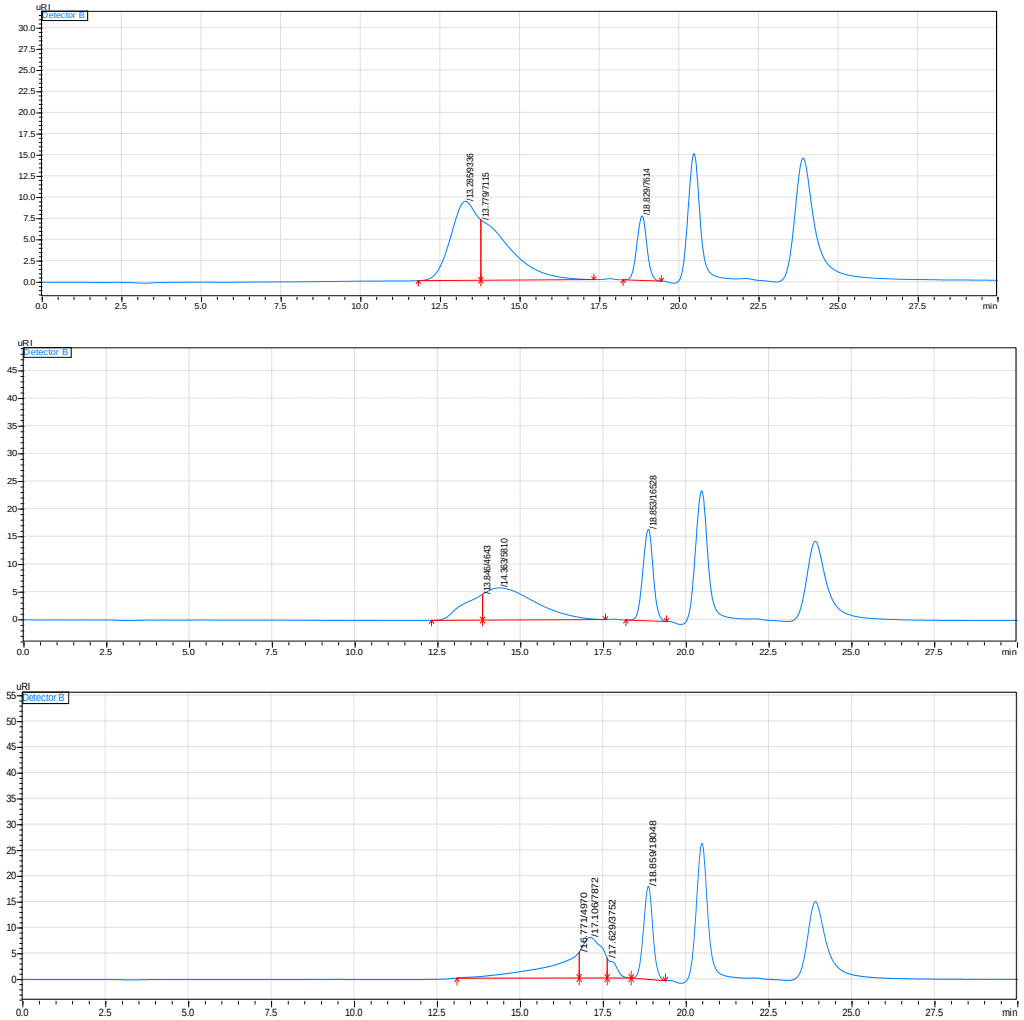


Figure 6.5: The change of molecular mass of the low viscosity alginate incubated with DS44 alginate lyase as analysed by HPLC. (A): control sample (0.5% alginate), (B): sample at 1h incubation, (C): sample after 24h incubation.

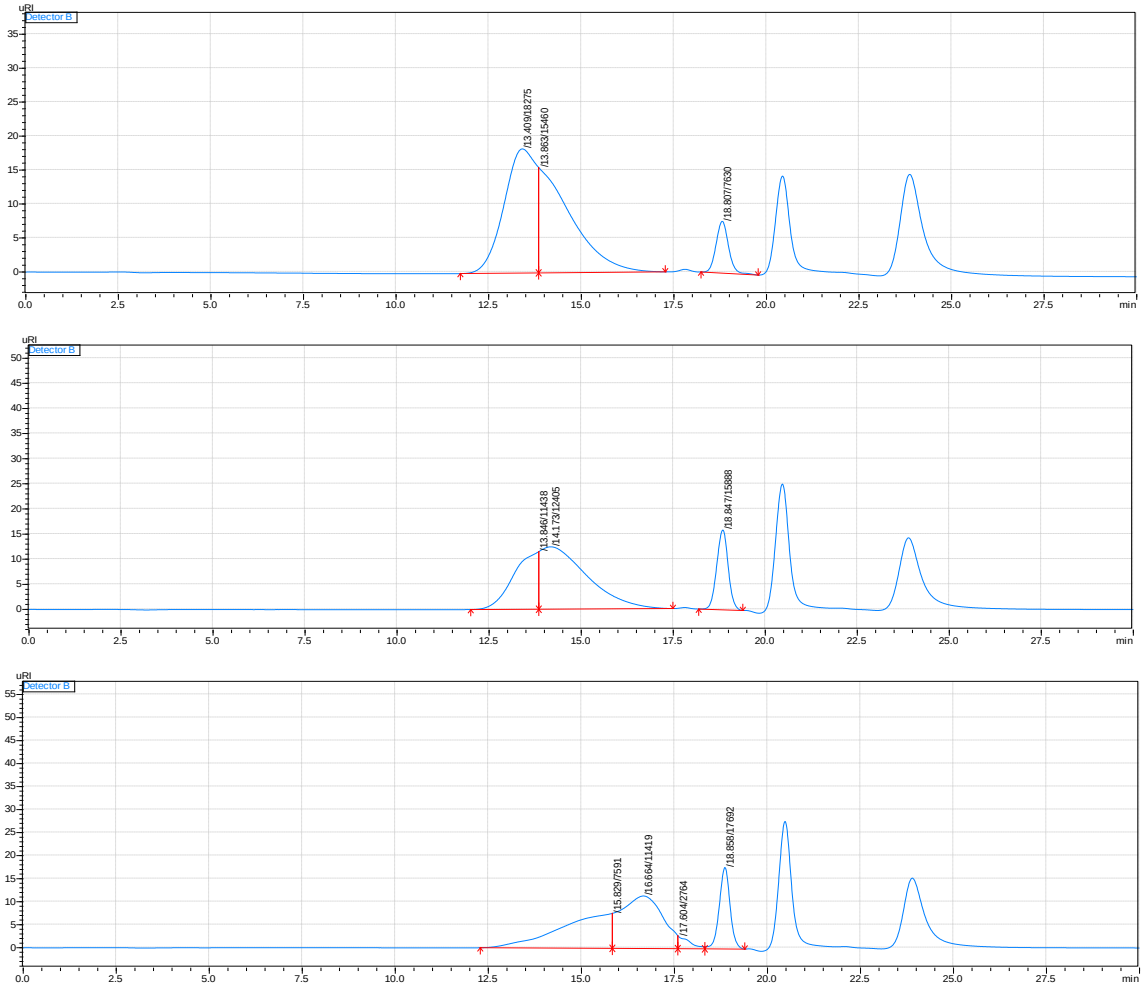


Figure 6.6: The change of molecular mass of the low viscosity alginate incubated with DS44 alginate lyase as analysed by HPLC. (A): control sample (1% alginate), (B): sample at 1h incubation, (C): sample after 24h incubation.

Table 6.3: The change of molecular weight of the low viscosity alginate of 0.5% and 1% after incubation with DS79 alginate lyase at 1h and 24h.

	Peak 1		Peak 2		Peak 3		Peak 4	
	%	MW (kDa)	%	MW (kDa)	%	MW (kDa)	%	MW (KDa)
0.5% the low viscosity alginate								
Control	46.3	552.0	39.4	305.0	14.3	0.7		
1h	24.0	94.6	27.5	17.8	24.0	12.6	3.1	2.4
24h	20.3	8.7	46.4	3.7	11.8	2.5	21.5	0.7
1% the low viscosity alginate								
Control	48.7	475.7	43.2	275.8	8.1	0.7		
1h	12.4	320.4	74.8	74.9	3.5	2.4	9.3	0.7
24h	31.8	8.1	43.9	3.7	12.4	2.7	11.9	0.7

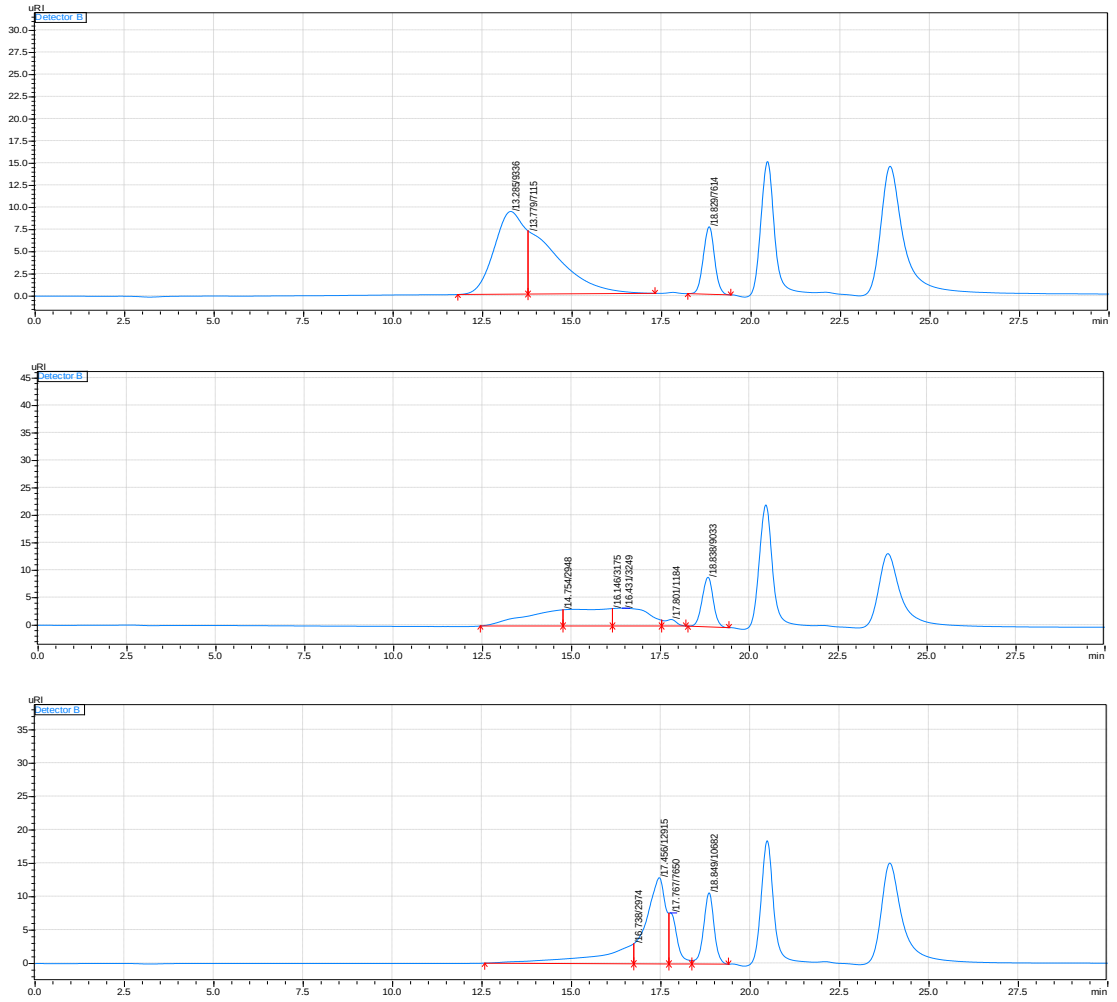


Figure 6.7: The change of molecular mass of the low viscosity alginate incubated with DS79 alginate lyase as analysed by HPLC. (A): control sample (0.5% alginate), (B): sample at 1h incubation, (C): sample after 24h incubation.

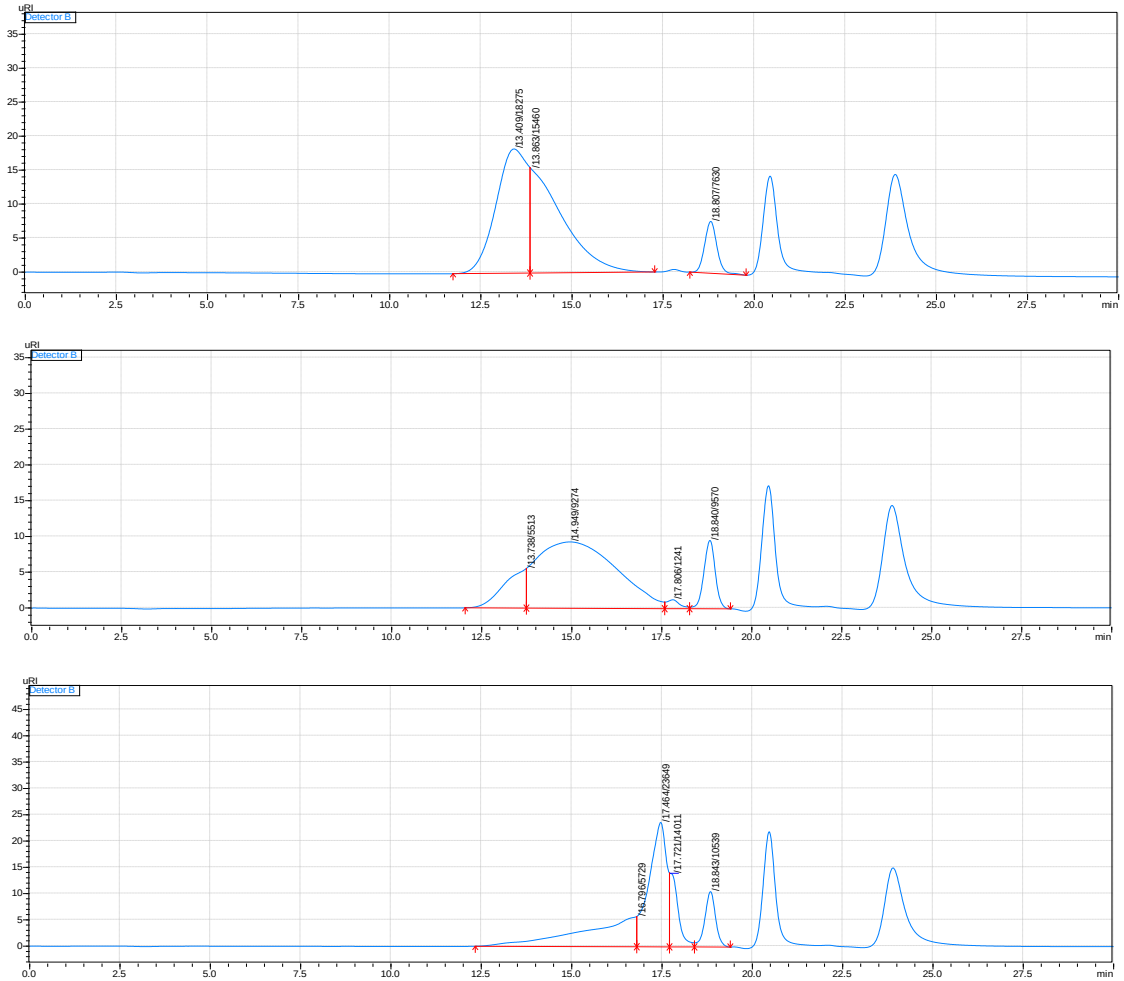


Figure 6.8: The change of molecular mass of the low viscosity alginate incubated with DS79 alginate lyase as analysed by HPLC. (A): control sample (1% alginate), (B): sample at 1h incubation, (C): sample after 24h incubation.

6.4 Conclusion

In conclusion, a technique for DNA screening from actinobacteria had been developed and validated to discover alginate lyases from actinobacteria from a range of natural sources. Based on this technique, the new structures and multiple bioactive functions of alginate oligosaccharides may be explored.

Furthermore, the recombinant alginate lyases showed high enzymatic activity on hydrolysis of commercial alginate, approximately 80% reduction within a 1 hour treatment and up to 98 % reduction within a 24 hours exposure. The enzymes from this study have the potential to produce ultra-low molecular weight and low viscosity alginate for specific applications.

CHAPTER 7: MAJOR FINDINGS AND FUTURE DIRECTIONS

The broad aims of this study were to screen, purify and characterise polysaccharide-degrading enzymes from actinobacteria growing on seaweed and identify novel enzymes. The hypothesis of this study that 'seaweed is a potential source of actinobacteria that produce novel polysaccharide-degrading enzymes' was proven, as degrading seaweed is a good substrate for the growth of actinobacteria which produced the enzymes with high degradation activity. This study has provided the initial information for further research in terms of basic and applied sciences. The major findings of this thesis are discussed in this chapter.

7.1 The major findings of this project

7.1.1 Seaweed is a good source of actinobacteria that yield polysaccharide-degrading enzymes.

To the best of our knowledge, this project was the first attempt to isolate actinobacteria from (South Australian) decomposing seaweed. This source yielded a large number of actinobacteria which could produce polysaccharide-degrading enzymes, up to 36% of the total isolates. Of these, three strains which showed the highest activity in breaking down alginate were studied further. All three belonged to the genus *Streptomyces* and contributed to increasing in the limited number of reported alginate lyase from *Streptomyces.* spp. (Kim *et al.*, 2009; Kim, 2010; Shin *et al.*, 2011). This study also used seaweed powder (2% Bull Kelp) as a substrate for the induction of alginate lyase production. Based on this data, the seaweed is recommended not only as a good source for actinobacteria that produced polysaccharide-degrading enzymes but also as a general substrate. There is an

abundance of natural seaweed in South Australia that can be used to produce high-value enzymes for seaweed bioprocessing.

7.1.2 Alginate lyase from 'seaweed' actinobacteria showed special characteristics in terms of alginate oligosaccharide degradation.

The alginate lyase from strains DS40, DS44, and DS79, which were identified as *Streptomyces griseorubens, Streptomyces luridiscabiei, Streptomyces sundarbansensis,* respectively, were purified and characterised. These enzymes have a molecular weight of approximately 29 kDa. DS44 and DS79 alginate lyases, which belonged to polysaccharide lyase family 7, could degrade both polyG and polyM blocks, indicated that they are bifunctional alginate lyase. They also displayed higher specific activity compared with other available alginate lyases (Yu *et al.*, 2018; Zhu *et al.*, 2018; Zhu *et al.*, 2016). Their degradation products were disaccharides, trisaccharides, and tetrasaccharides as endolytic alginate lyase. The optimal pH of both enzymes was 8.5 and optimal temperatures were 45^{o}C and 55^{o}C for DS44 and DS79 alginate lyases, respectively. They also were grouped into salt-tolerant alginate lyases and their activities increased in the presence of metal ions Mn^{2+}, Co^{2+}, and Fe^{2+}. The amino acid sequence of DS79 alginate lyase was almost similar to a reported enzyme (ALG-5 lyase from *Streptomyces* ALG-5 (Kim *et al*., 2009)), whereas the DS44 alginate lyase showed more than 10% difference, demonstrated the potential for efficient production of alginate oligosacharides with low degrees of polymerization.

7.1.3 Recombinant alginate lyase can be produced as a commercial enzyme.

In this study, the alginate lyase genes from strains DS44 and DS79 were cloned and expressed in *E. coli* BL21 (DE3) with pColdI expression vector. The recombinant enzymes were purified effectively by using Ni^{2+} Sepharose affinity chromatography and retained their biological activity. This helps to develop stable biocatalysts for the large scale preparation of alginate oligosaccharides. The recombinant alginate lyases can also meet the growing demand for commercial enzymes used in many medical and biotechnological applications (Dumorné & Severe, 2018; Inoue, 2018; Wong *et al.*, 2000).

7.1.4 The new primers encoding alginate lyase can be applied to screen alginate lyase from actinobacteria from various natural sources.

The primers including forward (5'-GCGTGGACATCAAGGAGAAC-3') and reverse (5'-AYCGGGMWAKGTGCGAATS-3') were effective in screening for the presence of alginate lyase genes from actinobacteria from different natural sources. Based on this, the novel alginate lyase, as well as the new structures and multiple bioactive functions of alginate oligosaccharides, can be further investigated extensively and easily.

7.2 Future directions

7.2.1 Screening fucoidanase using different types of seaweed.

In the research plan of this thesis, the actinobacteria were screened for their ability to produce polysaccharide-degrading enzymes that can degrade alginate and fucoidan. However, the results in Table 3.2 showed that there was very low activity against fucoidan. No single band from all peak fractions after the purification steps was obtained on the SDS-PAGE gel to indicate possible fucoidan degrading enzyme (data was not shown). Therefore, no further studies could be done due to no detected fucoidanase activity. The possible reason is that Bull Kelp has a low content of fucoidan (5-10%) compared with a higher content of 55 – 60% alginate. Based on this, further study needs to be carried out on the potential actinobacteria that can produce a range of polysaccharide-degrading enzymes, especially fucoidanase. The other types of seaweed such as *Fucus vesiculosus, Macrocystis pyrifera,* and *Undaria pinnatifida* should be used as carbon sources to induce the production of fucoidanase. Additionally, the fucoidan from a commercial supplier was analysed for its monosaccharides and the result showed that there was only approximately 48% fucose indicating for fucoidan, the other 42% belong to glucose indicating the presence laminarin (Appendix F), therefore, the fucoidan substrate used in DNS assay should have as high a purity as possible to make sure that the results are only due to fucoidanase activity. If the fucoidanase can be found, it will be processed using the same steps as the alginate determination including purification and characterisation to discover potentially novel fucoidanase from marine actinobacteria. Moreover, they also can be cloned and expressed in *E. coli* to produce the enzymes on a large scale.

7.2.2 Improvement of soluble form of recombinant alginate lyase

From the expression results in section 5.3.2, the DS44 and DS79 recombinant alginate lyases were expressed as insoluble inclusion bodies. The major problem of insoluble protein is that they have to be refolded correctly to show the biocatalytic of the recombinant enzymes. However, due to time constraints, it was not feasible to achieve an increase in the amount of soluble expressed protein. There are some possible solutions such as changing induction conditions, incubation time, the *E. coli* host strain or the extraction method. In detail, the different times of induction should be tried between the early and late logarithmic phases. The incubation time can be longer than 24 hours at 15^{o}C. The commercial strain of host *E. coli* which is Origami competent cells from Merck Millipore, can facilitate solubilisation of expressed proteins. Besides, using co-expression of a molecular chaperone is also an effective way to solve this problem (Schlieker *et al.*, 2002). The Chaperone Plasmid Set (Cat.#3340) combined with pCold vector was recommended by Takara-Bio. The various chaperons including pG-KJE8, pGro7, pKJE7, pGTf2, and pTF16 should be investigated to determine their optimal levels in the expression of target proteins (Shin *et al.*, 2011). Finally, it is also effective to change the extraction method using sonication with 0.1 to 1% of detergents such as Octylglycoside, Nonidet P-40, Tripton X-100, or 10 mg/ml of Lysozyme.

7.2.3 Promising applications of alginate lyase

7.2.3.1 Commercial enzyme productions

Through the successful alginate lyase production and enzyme purification of this study, highly efficient DS44 and DS79 recombinant alginate lyases were used to

further reduce the molecular weight of commercial sodium alginate in order to obtain new alginate compounds with various functionalities. Therefore, the bioreactor studies should be investigated to scale up the fermentation process for the mass production of the recombinant alginate lyases. Together with a variety available commercial sodium alginate, a range of new oligoalginates with different molecular weight will be produced using the alginate lyase as a bio-catalyst and their bio-functions will be explored.

7.2.3.2 Development of novel alginate based marine bio-degradable and bio-composite materials

One of the major directions the future direction is to utilise the alginate lyases selected from this study and develop commercial applications of novel bio-composite made with enzymatic hydrolysed sodium alginate for packaging materials with potential marine biodegradability.

As of 2015, the cumulative global plastic production on a global scale exceeded 8.3 billion metric tons and as the result of 6.3 billion metric tons became wastage. It is estimated that by 2050 this figure will increase to an amount of 12 billion metric tons (Parker, 2018). Only 21% of plastic waste, amounting to 6.3 billion metric tons was recycled or incinerated, which resulted in 79% entering landfill sites or being uncontrolled and released into the environment. Many plastics take longer than 100 years to fully degrade, highlighting the need to either reduce plastic use or use degradable bioplastics. Among them, 4.8 to 12.7 million tons land-based plastic debris escaped into the ocean every year and resulting in about 80 % of marine litter consists of plastic (Jambeck *et al.*, 2015). This result in the death of marine organisms due to the entanglement and ingestion by the plastic waste. Besides,

there are many chemical additives in plastics. These microplastics, nanoplastics and chemicals can cause various harmful effects to marine life, such as hormone disruption and reproductive abnormalities. Therefore, bioplastics are produced by using bio-based polymers from biomass and have begun to be commercially used in many industries. However, despite the urgent need for sustainable and biodegradable materials for mass-produced commercial products, the use of biopolymers and their composites in modern engineering and industry settings there remain many limitations.

Sodium alginate based novel bio-composite materials had been widely studied especially for food packaging application. Along the backbone, alginate has several free carboxyl and hydroxyl groups, which as a result make it a good candidate for chemical functionalisation for the alteration of interesting properties such as hydrophobicity, solubility, biological, and physicochemical characteristics. A preliminary study of developing novel bio-composite made with enzymatic hydrolysed sodium alginate for packaging materials had been carried using the DS44 and DS79 recombinant alginate lyases and the results had demonstrated the feasibility of potential marine biodegradability of each biomaterial.

- **Materials, methods and pilot studies**

In this study, a novel Ca-alginate/carrageenan bio-composite materials was developed by mixing 1% of low molecular weight commercial sodium alginate with 1% commercial carrageenan in a solution and oven dried to a biofilm. After soaking the biofilm in 1% $CaCl_2$ solution, Ca-alginate/carrageenan cross-linking was achieved, and the biofilm became stronger and waterproof (Figure 7.1).

Figure 7.1 Ca-alginate/carrageenan biofilm

Furthermore, the potential marine biodegradability of the Ca-alginate/carrageenan biofilm was tested by incubating the biofilm in natural seawater with the addition of actinobacteria (strains DS44 and DS79) that produces polysaccharide-degrading enzymes. After 14 days of incubation at 27°C, the Ca-alginate/carrageenan biofilm was degraded up to 80% by weight (Figure 7.2).

- **Future plans**

In the next step, a more systemic investigation of potential commercial applications of the novel bio-composite made will be conducted with enzymatic hydrolysed sodium alginate for packaging materials. Different molecular weight alginates will be selectively produced through controlled enzymatic degradation, in order to develop bio-materials or bio-plastics with more functions, such as antimicrobial activities, various physical properties, in including density, strength, and flexibly.

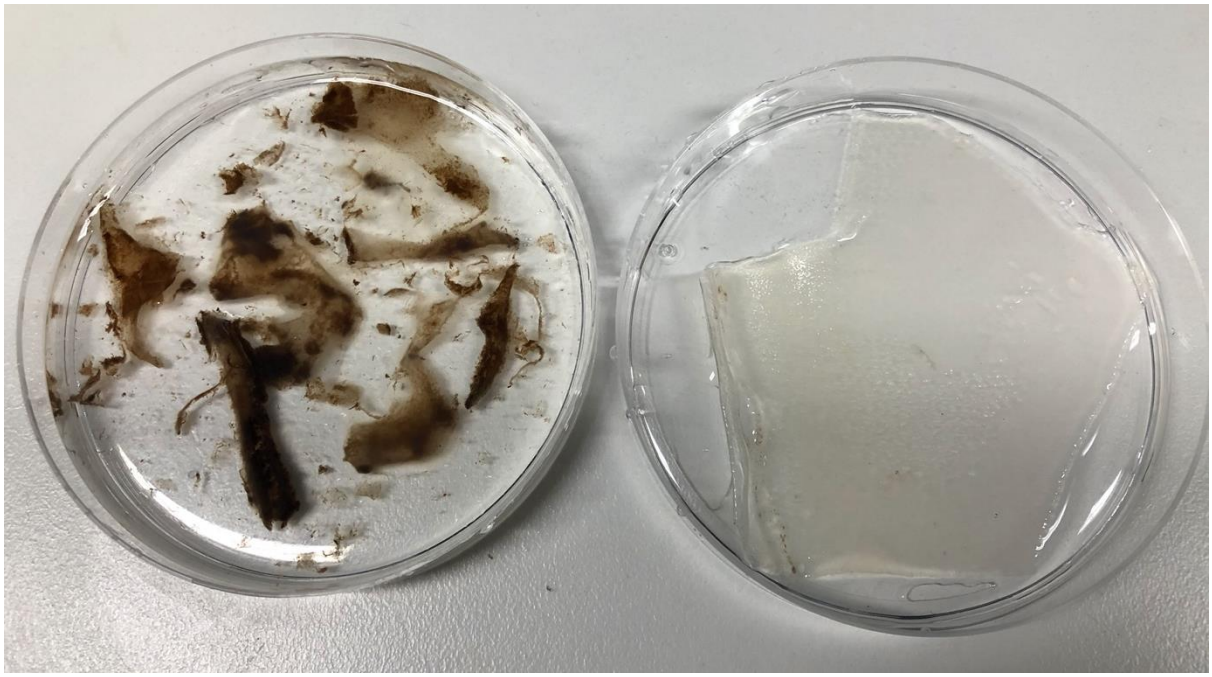


Figure 7.2 Comparison of Ca-alginate/carrageenan biofilm degradation in natural seawater with addition of actinobacteria that produces polysaccharide-degrading enzymes (left) and the control (right).

7.2.3.3 Potential antimicrobial function of various molecular weight alginate for different applications

The antimicrobial activity of alginate with particular structure had been reported widely (Friedman *et al.*, 2013; Kumar *et al.*, 2019; Oussalah *et al.*, 2006; Park *et al.*, 2016; Patel *et al.*, 2019; Salem *et al.*, 2019; Sen *et al.*, 2017; Shankar & Rhim, 2018; Tang *et al.*, 2018). The study of enzymatic hydrolysed sodium alginate from DS44 and DS79 alginate lyase can be carried out by using HPLC to obtain various molecular weight oligosaccharides that can be experimented on antimicrobial activity.

7.2.3.4 Development of alginate based 3D bioprinting materials and functional pharmaceutical alginate for drug delivery

In the recent years, the demand for alginate-based biomaterials used in tissue engineering and drug delivery has increased significantly (Axpe & Oyen, 2016; Datta *et al.,* 2019; Liu *et al.*, 2018). Alginate hydrogel with a complex structure and high shape fidelity is a vital material for preparing bioinks and 3D printed scaffolds for tissue engineering (Datta *et al.,* 2019). The viscosity of alginate-based bioink determines the shape fidelity and structure of hydrogels. There are many factors affect its viscosity including alginate concentration, alginate molecular weight and density of cells which should be optimised. For example, the laser-assisted bioprinting requires bioinks with viscosities of 1 and 300 mPa · s and cell densities of 108 cells/ml. Therefore, the structural and mechanical characteristics of alginate are essential for printed tissue.

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Figure 7.3: Composition of alginate bio-ink. (This figure has been reproduced from Axpe & Oyen (2016).

On the other hand, alginate has been used as a carrier for the encapsulation and immobilisation of cells, drugs, proteins, and bioactive molecules due to its biodegradable and biocompatible properties (Gudapati *et al.*, 2014; Yanez *et al.*, 2015).There are many reports of the microspheres, porous scaffolds, and alginate-based hydrogel blends for drug delivery in various fields of tissue engineering (Cui *et al.*, 2009; Freier *et al.*, 2005)

In summary, enzymatically degradable alginates that present suitable characteristics will be investigated, in order to assist in inventing advanced materials for 3D bioprinting and drug delivery technology.

APPENDIX A

1. Media for Isolation Actinomycetes from seaweed

1.1. Modified Humic Acid vitamin B agar (HVA)

Per litre RO water	
Humic acid	1 g
Na_2HPO_4	0.25 g
KCl	0.85 g
$MgSO_4.7H_2O$	0.025 g
$FeSO_4.7H_2O$	0.05 g
$CaCO_3$	0.01 g
Agar	18 g
Vitamin B 100x (added after media autoclaved)	1 ml

Adjust pH to 7.2 ± 0.2

Dilute humic acid in 0.2 M NaOH before adding it into the media solution as it will not dissolve completely in water.

Vitamin B (100x) per 100 ml RO water	
Thiamine-hydrochloride	5 mg
Riboflavin	5 mg
Niacin	5 mg
Pyridoxine-hydrochloride	5 mg
Inositol	5 mg
Ca-panthotenate	5 mg
p-aminobenzoic acid	25 mg
Biotin	25 mg

1.2. Glycerol asparagine agar (GAA)

Per litre RO water	
Glycerol	10 ml
L-asparagin	1 g
K_2HPO_4	1 g
Trace salt solution	1 ml
Agar	18 g

Adjust pH to 7.2 ± 0.2

Trace salt solution per 100 ml RO water	
$FeSO_4.7H_2O$	0.1 g
$MnCl_2.4H_2O$	0.1 g
$ZnSO_4.7H_2O$	0.1 g

1.3. Starch yeast peptone agar (SYP)

Per litre RO water	
Starch	10 g
Yeast extract	4 g
Peptone	2 g
Agar	18 g

Adjust pH to 7.2 ± 0.2

2. Media for actinobacteria purification

Half-strength Potato Dextrose Agar (HPDA)

Per litre sea water	
PDA (Oxoid)	19.5 g
Agar	7.5 g

Adjust pH to 7.2 ± 0.2

3. Inoculum medium (IM22)

Per litre RO water	
Glucose	15 g
Calcium carbonate	2 g
Sodium chloride	5 g
Soyatone	15 g
Parmamedia	5 g

Adjust pH to 7.2 ± 0.2

4. Media for culturing *E. coli*

4.1. Luria-Bertani (LB) medium

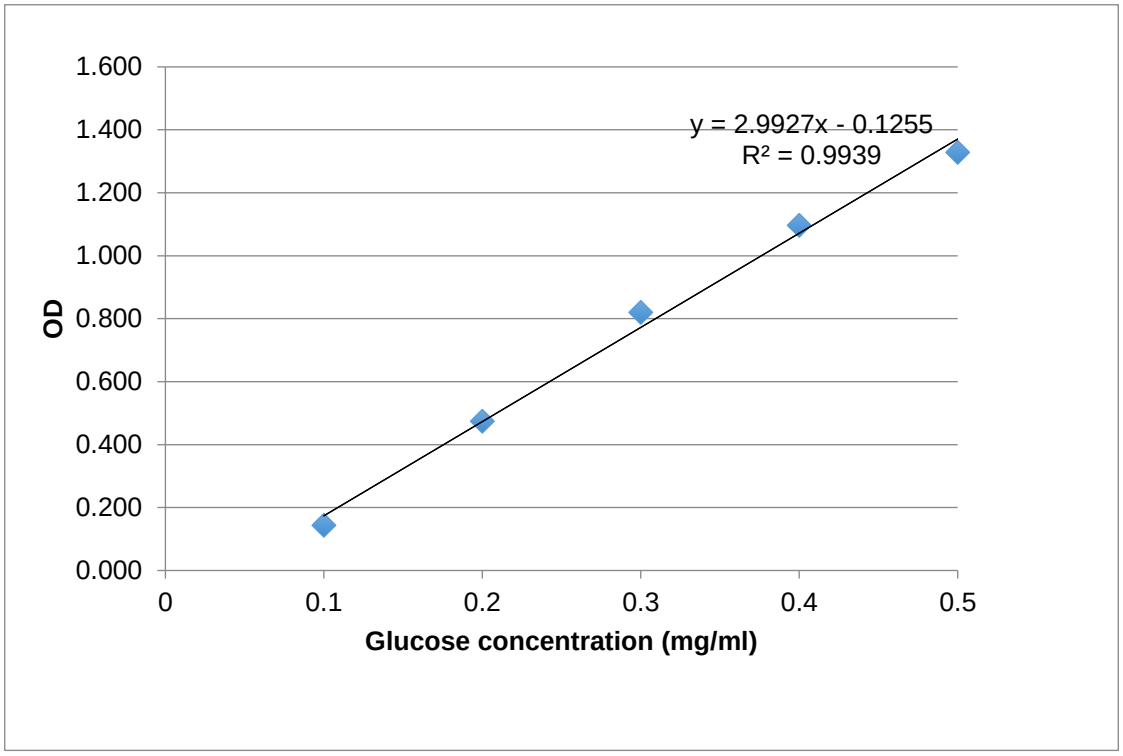
Per litre RO water	
Tryptone	10 g
Yeast extract	5 g
NaCl	5 g

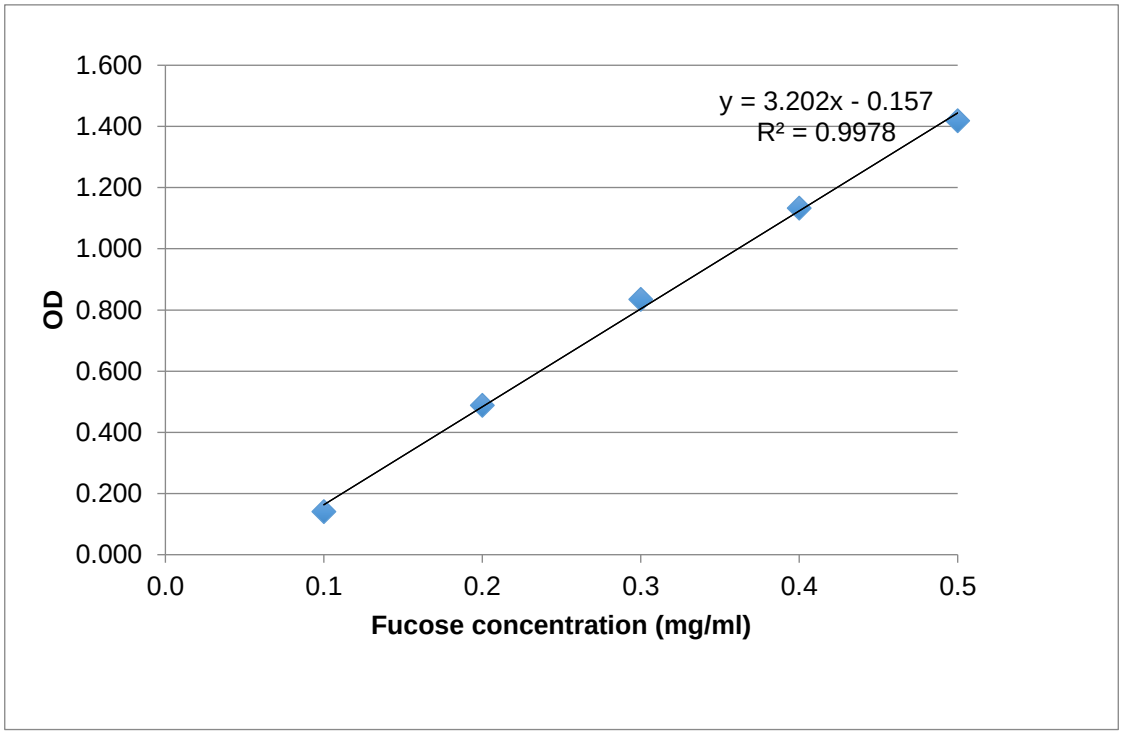
4.2. Luria-Bertani (LB) agar

Per litre RO water	
Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Agar	12 g

APPENDIX B

1. Standard curve of DNS assay

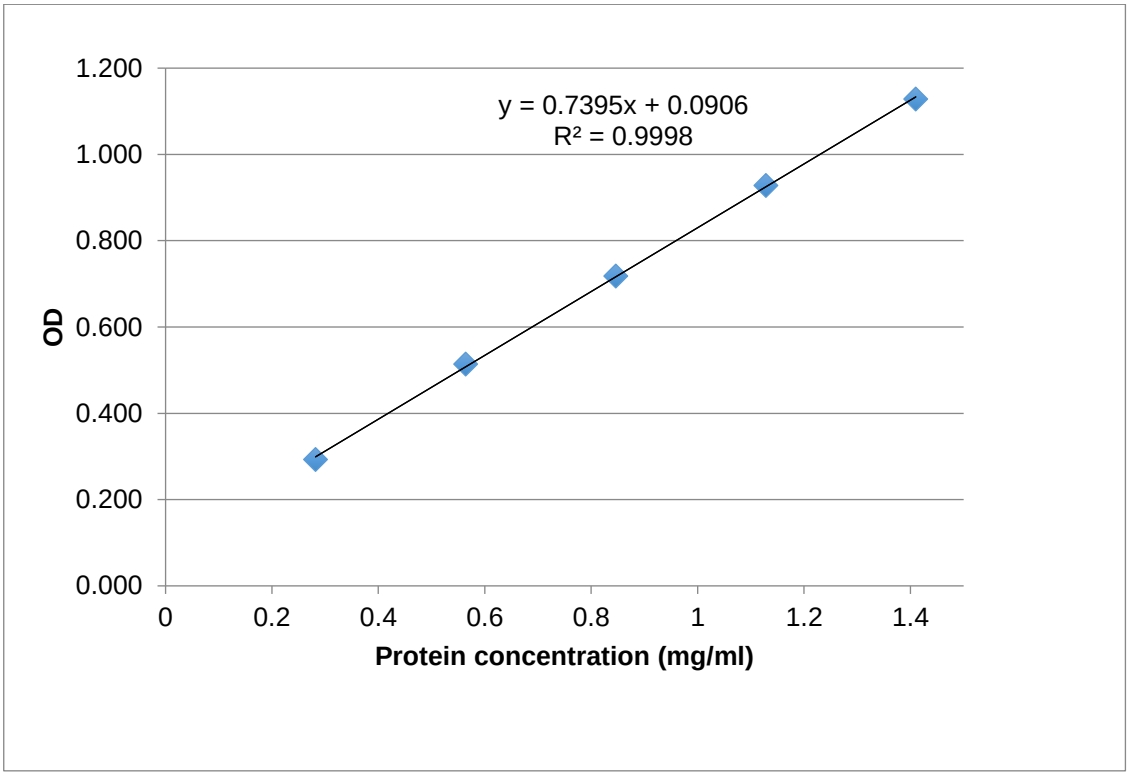




2. Protein standard dilution (BCA assay).

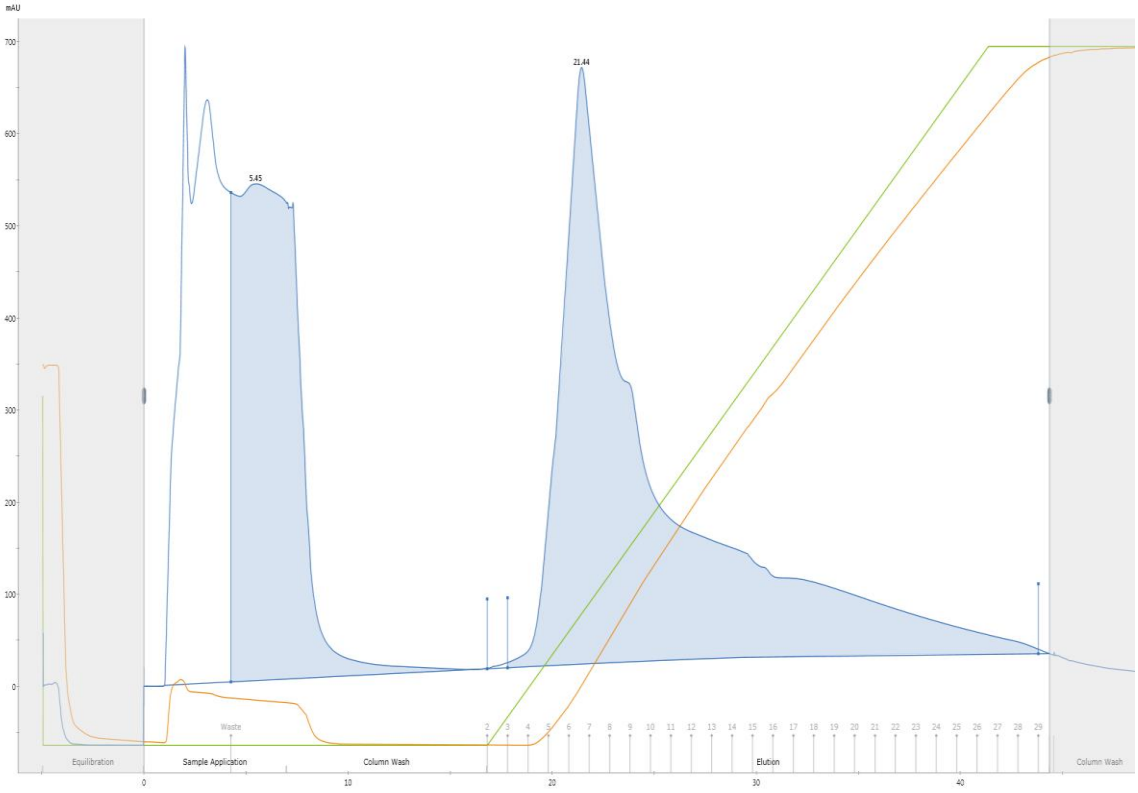
Concentration (mg/ml)	Percentage (%)	Volume of previous standard (µl)	Volume of MQ water (µl)
1.41	100	50 (1.41 mg/mL stock)	0
1.128	80	25	25
0.846	60	25	25
0.564	40	20	30
0.282	20	25	25
0.141	10	25	25
0	0	20	30

3. Standard curve of BCA assay

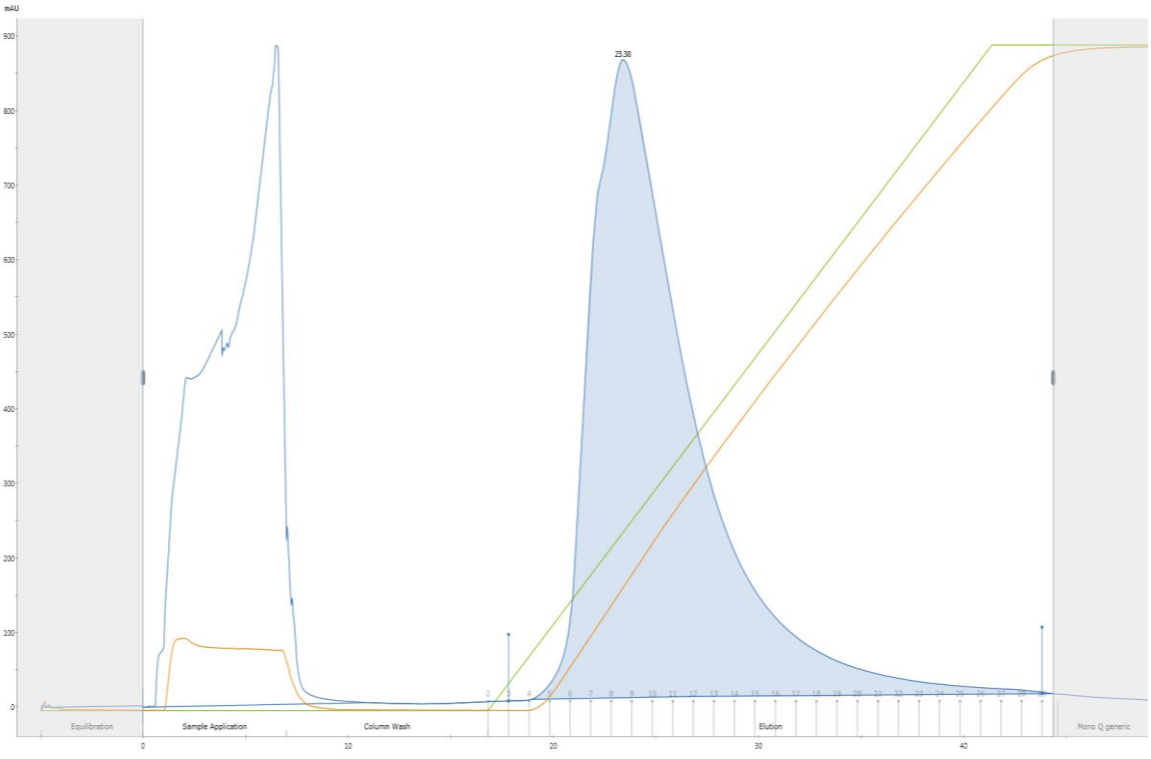


APPENDIX C

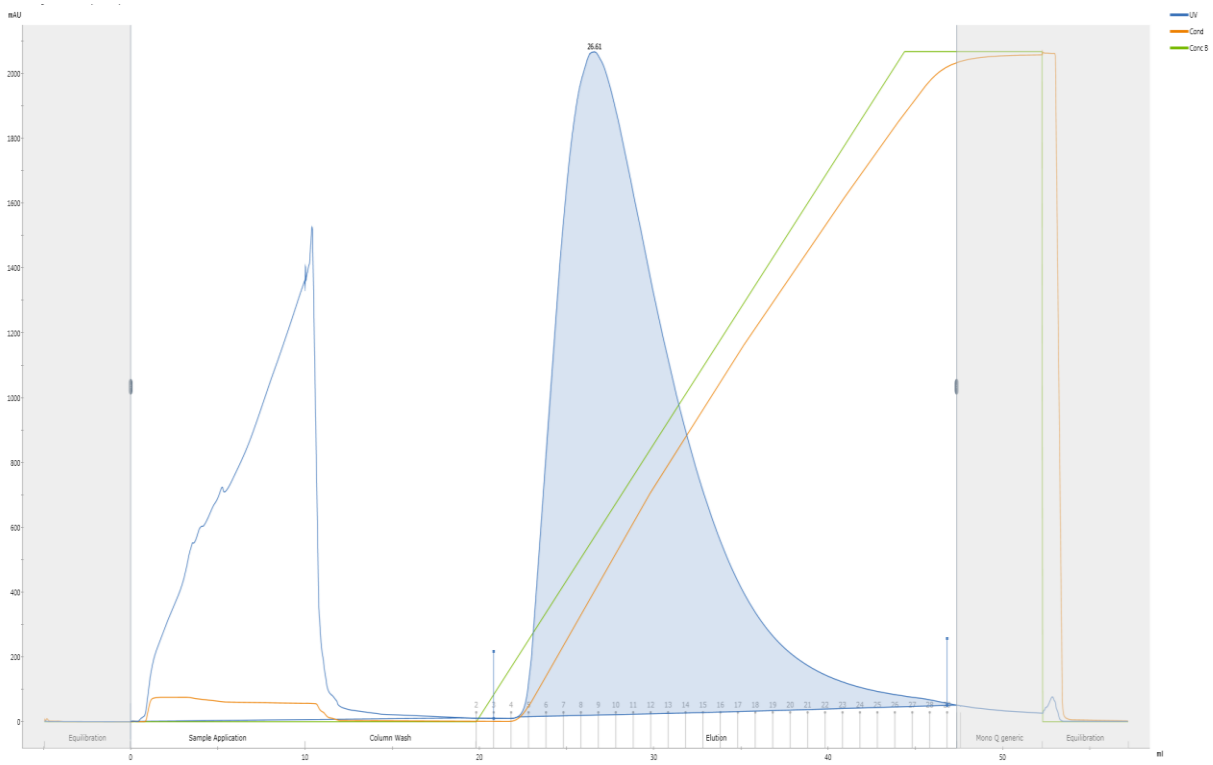
1. Anion exchange chromatography



DS40

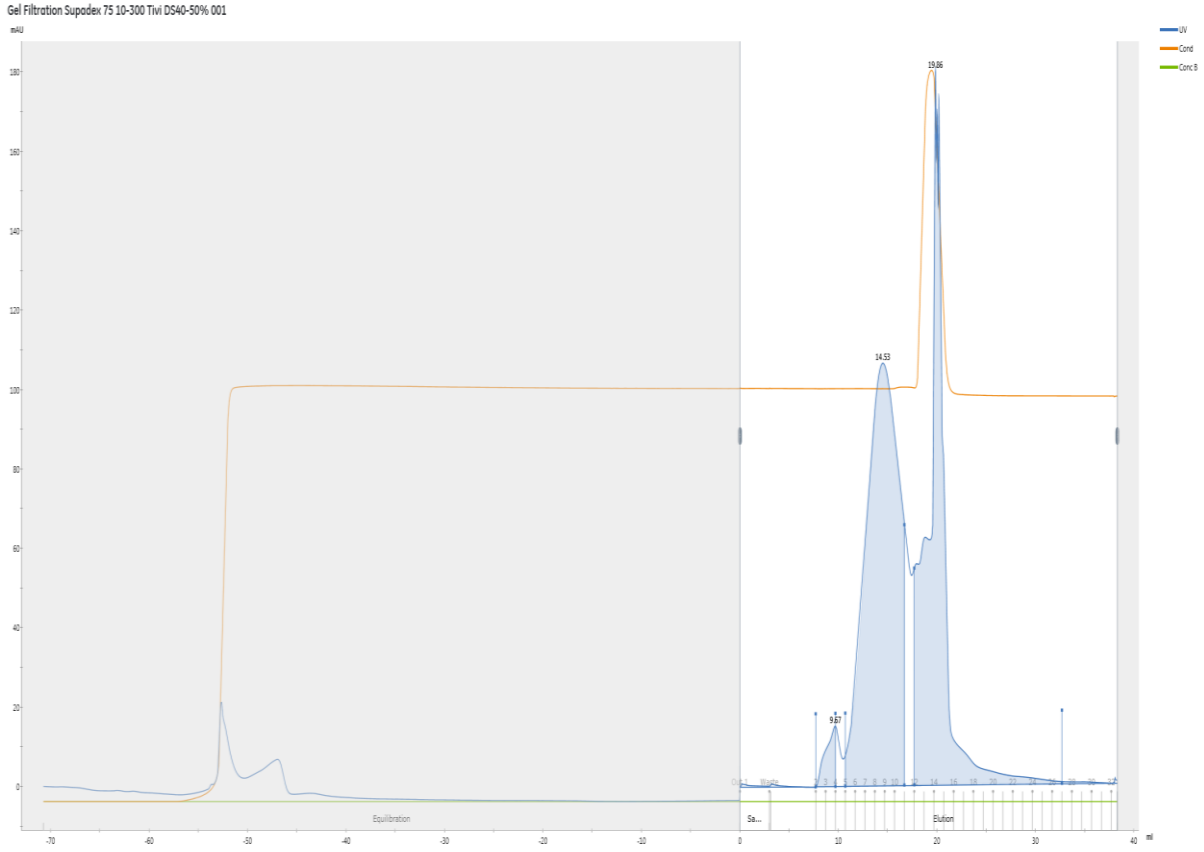


DS44

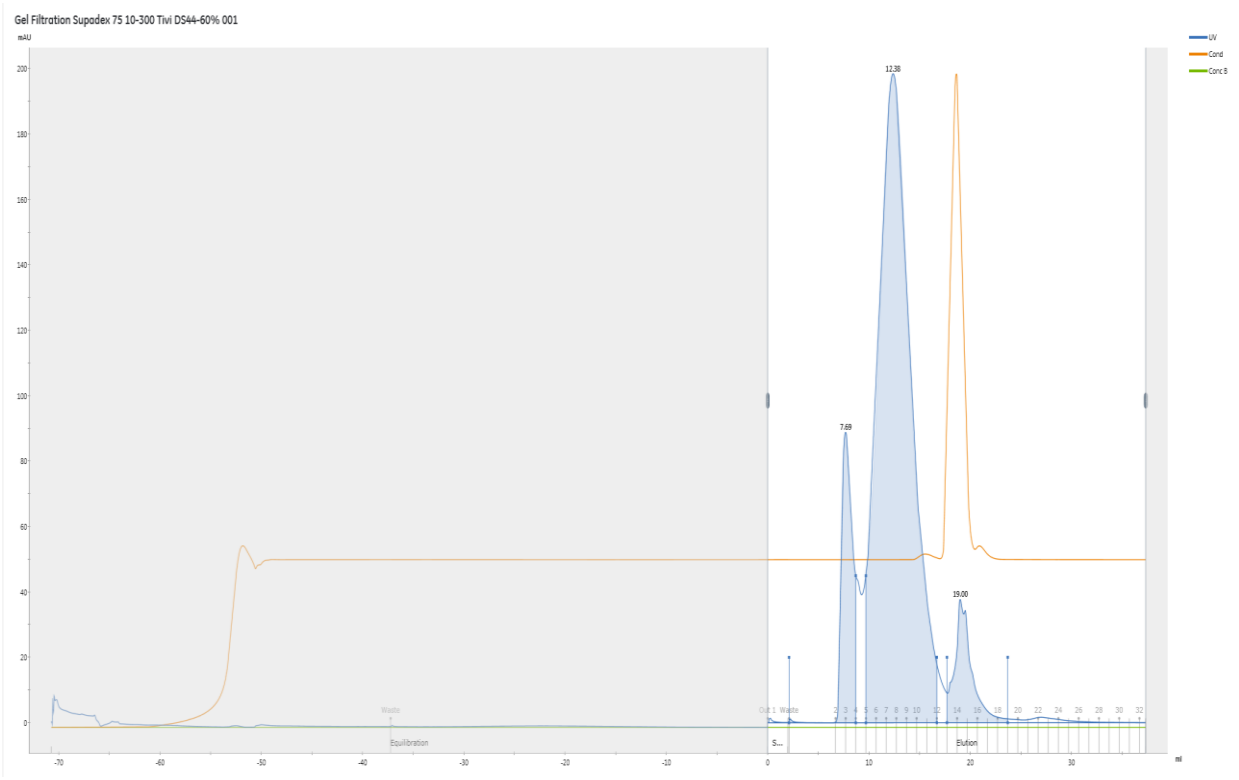


DS79

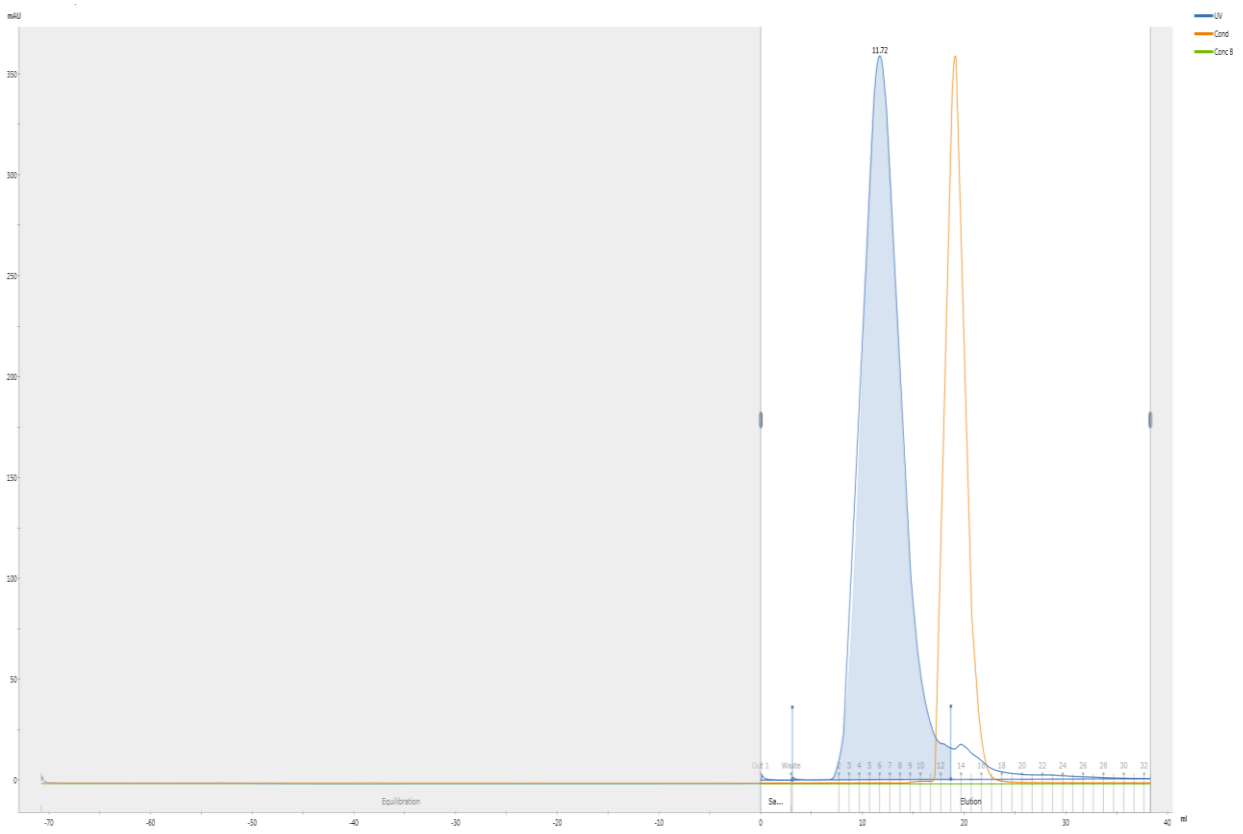
2. Size exclusion chromatography



DS40


Gel Filtration Supadex 75 10-300 Tivi DS44-60% 001
mAU
UV
Cond
Conc B
12.38
7.69
19.00
Waste
Equilibration
Elution
ml

DS44


mAU
UV
Cond
Conc B
11.72
Waste
Equilibration
Elution
ml

DS79

APPENDIX D

1. SDS – PAGE buffer

1.1. 10X Running buffer

0.05 M Tris

1.92 M Gycine

0.6% (w/v) SDS

Solution was diluted 1:10 to get a 1 x running buffer

1.2. 4X SDS loading buffer (10ml)

0.5 M Tris - HCl	5 ml
Glycerol	4 ml
SDS	0.8 g
DTT (added fresh)	0.62 g
Saturated bromophenol blue	2 µl

SDS loading buffer was made up to 10 ml with RO water and divided into 1 ml aliquots for storage at -20^{o}C.

2. Gel electrophoresis buffer

2.1. 10X TBE electrophoresis buffer

Tris base	108 g
Boric acid	55 g
EDTA	9.3 g
MQ water	1 L

2.1. 20X Sodium Borate (SB) electrophoresis buffer (pH 8.0)

Boric acid	48 g
NaOH	8 g
MQ water	1 L

3. Coomassie blue stain

Coomassie Brilliant Blue R-250	2 g
Methanol	20%
Glacial acetic acid	5%
MQ water	1L

4. Bacterial lysis buffer

SDS	2 g
Beta Mercaptoethanol (BME)	10 g
MQ water	100 ml

APPENDIX E

1. AccuPrep® Nano-Plus Plasmid DNA Extraction Kit (Bioneer)

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2. QIAquick gel extraction kit

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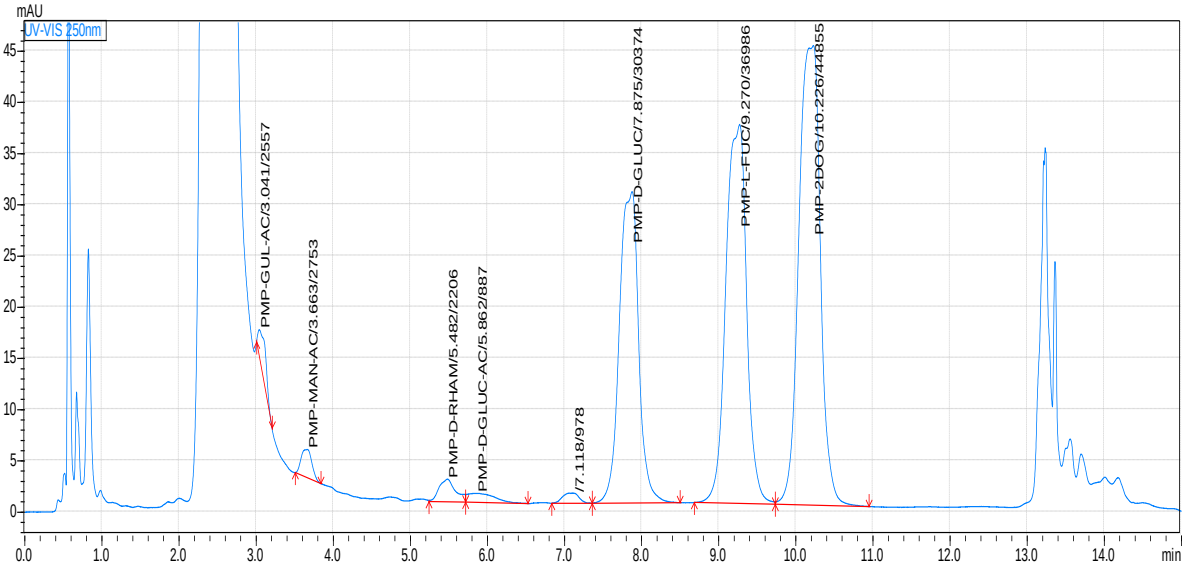
APPENDIX F

Monosaccharide analysis of fucoidan from Marinova

Peak#	Ret. Time	Name	Conc.	Area	%
1	3.041	PMP-GUL-AC	0	25204	3.0
2	3.663	PMP-MAN-AC	0	26475	2.3
3	5.482	PMP-D-RHAM	16.904	33154	2.7
4	5.862	PMP-D-GLUC-AC	12.087	21890	1.9
5	7.118		0	14583	0.0
6	7.875	PMP-D-GLUC	268.797	529949	41.9
7	9.27	PMP-L-FUC	305.466	692835	48.1
8	3.041	PMP-GUL-AC	0	25204	3.0
Total			603.254	2217585	100



REFERENCES

Acarturk, F & Takka, S, 1999, 'Calcium alginate microparticles for oral administration: II. Effect of formulation factors on drug release and drug entrapment efficiency', *J Microencapsul*, vol. 16, no. 3, pp. 291-301.

Aguilar, O, Albiter, V, Serrano-Carreón, L & Rito-Palomares, M, 2006, 'Direct comparison between ion-exchange chromatography and aqueous two-phase processes for the partial purification of penicillin acylase produced by E. coli', *Journal of Chromatography B*, vol. m835, no. 1–2, pp. 77-83.

Alanazi, M, 2013, 'Isolation, characterisation and antimicrobial activity of endophytic actinobacteria from South Australia seaweed', Masters thesis, Department of Medical Biotechnology, Flinders University.

Albrecht, MT & Schiller, NL, 2005, 'Alginate lyase (AlgL) activity is required for alginate biosynthesis in Pseudomonas aeruginosa', *J Bacteriol*, vol. 187, no. 11, pp. 3869-72.

Ale, MT & Meyer, AS, 2013, 'Fucoidans from brown seaweeds: an update on structures, extraction techniques and use of enzymes as tools for structural elucidation', *RSC Advances*, vol. 3, no. 22, pp. 8131-41.

An, QD, Zhang, GL, Wu, HT, Zhang, ZC, Zheng, GS, Luan, L, Murata, Y & Li, X, 2009, 'Alginate-deriving oligosaccharide production by alginase from newly isolated Flavobacterium sp. LXA and its potential application in protection against pathogens', *J Appl Microbiol*, vol. 106, no. 1, pp. 161-70.

Andrew, SM, Titus, JA & Zumstein, L, 2001, 'Dialysis and concentration of protein solutions', *Curr Protoc Immunol*, vol. Appendix 3, p. Appendix 3H.

Andriamanantoanina, H & Rinaudo, M, 2010, 'Characterization of the alginates from five Madagascan brown algae', *Carbohydr Polym*, vol. 82, no. 3, pp. 555-60.

APCAP, 2012, 'Seaweads Australia', in *8th Asia Pacific Conference on Algal Biotechnology*, Adelaide.

Anteneh, Y, 2020, 'Isolation, diversity and antimicrobial activities of marine sponge associated bacteria of South Australia', Doctoral thesis, Department of Medical Biotechnology, Flinders University.

Axpe, E & Oyen, ML, 2016, 'Applications of Alginate-Based Bioinks in 3D Bioprinting', *Int J Mol Sci*, vol. 17, no. 12, p. 1976.

Bakare, MK, Adewale, IO, Ajayi, A & Shonukan, OO, 2005, 'Purification and characterization of cellulase from the wild-type and two improved mutants of Pseudomonas fluorescens', *African Journal of Biotechnology*, vol. 4, no. 9, pp. 898-904.

Bakunina, I, Nedashkovskaia, OI, Alekseeva, SA, Ivanova, EP, Romanenko, LA, Gorshkova, NM, Isakov, VV, Zviagintseva, TN & Mikhailov, VV, 2002, 'Degradation of fucoidan by the marine proteobacterium Pseudoalteromonas citrea', *Mikrobiologia*, vol. 71, no. 1, pp. 49-55.

Bakunina, I, Shevchenko, LS, Nedashkovskaia, OI, Shevchenko, NM, Alekseeva, SA, Mikhailov, VV & Zviagintseva, TN, 2000, 'Screening of marine bacteria for fucoidan hydrolases', *Mikrobiologiia*, vol. 69, no. 3, pp. 370-6.

Baneyx, F, 1999, 'Recombinant protein expression in Escherichia coli', *Current Opinion in Biotechnology*, vol. 10, no. 5, pp. 411-21.

Berg, JM, Tymoczko, JL & Stryer, L, 2002, *Biochemistry*, 5th edn, New York: W H Freeman.

Berteau, O & Mulloy, B, 2003, 'Sulfated fucans, fresh perspectives: structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide', *Glycobiology*, vol. 13, no. 6, pp. 29R-40R.

Bilan, MI, Grachev, AA, Ustuzhanina, NE, Shashkov, AS, Nifantiev, NE & Usov, AI, 2002, 'Structure of a fucoidan from the brown seaweed Fucus evanescens C.Ag', *Carbohydr Res*, vol. 337, no. 8, pp. 719-30.

Bilan, MI, Kusaykin, MI, Grachev, AA, Tsvetkova, EA, Zvyagintseva, TN, Nifantiev, NE & Usov, AI, 2005, 'Effect of enzyme preparation from the marine mollusk Littorina kurila on fucoidan from the brown alga Fucus distichus', *Biochemistry (Mosc)*, vol. 70, no. 12, pp. 1321-6.

Bizuye, A, Moges, F & Andualem, B, 2013, 'Isolation and screening of antibiotic producing actinomycetes from soils in Gondar town, North West Ethiopia', *Asian Pacific Journal of Tropical Disease*, vol. 3, no. 5, pp. 375-81.

Bodzon-Kulakowska, A, Bierczynska-Krzysik, A, Dylag, T, Drabik, A, Suder, P, Noga, M, Jarzebinska, J & Silberring, J, 2007, 'Methods for samples preparation in proteomic research', *Journal of Chromatography B*, vol. 849, no. 1–2, pp. 1-31.

Bornhorst, JA & Falke, JJ, 2000, 'Purification of proteins using polyhistidine affinity tags', *Methods Enzymol*, vol. 326, pp. 245-54.

Boyen, C, Bertheau, Y, Barbeyron, T & Kloareg, B, 1990, 'Preparation of guluronate lyase from *Pseudomonas alginovora* for protoplast isolation in Laminaria', *Enzyme Microb. Technol.*, no. 12, pp. 885 - 900.

Bredholt, H, Fjærvik, E, Johnsen, G & Zotchev, SB, 2008, 'Actinomycetes from sediments in the Trondheim fjord, Norway: diversity and biological activity', *Mar Drugs*, vol. 6, no. 1, pp. 12-24.

Brown, BJ & Preston, JF, 1991, 'L-guluronan-specific alginate lyase from a marine bacterium associated with Sargassum', *Carbohydrate research*, vol. 211, no. 1, pp. 91-102.

Budarin, VL, Zhao, Y, Gronnow, M, Shuttleworth, P, Breeden, S, Macquarrie, D & Clark, J, 2011, 'Microwave-mediated pyrolysis of macro-algae', *Green Chem*, vol. 13, pp. 2330 - 3.

Busso, D, Delagoutte-Busso, B & Moras, D, 2005, 'Construction of a set Gateway-based destination vectors for high-throughput cloning and expression screening in *Escherichia coli*', *Anal Biochem*, vol. 343, no. 2, pp. 313-21.

Cao, L, Xie, L, Xue, X, Tan, H, Liu, Y & Zhou, S, 2007, 'Purification and characterization of alginate lyase from *Streptomyces* species strain A5 isolated from banana rhizosphere', *Journal of Agricultural and Food Chemistry*, vol. 55, no. 13, pp. 5113-7.

Chen, YY, Lin, SY, Yeh, YY, Hsiao, HH, Wu, CY, Chen, ST & Wang, AH, 2005, 'A modified protein precipitation procedure for efficient removal of albumin from serum', *Electrophoresis*, vol. 26, no. 11, pp. 2117-27.

Chevolot, L, Foucault, A, Chaubet, F, Kervarec, N, Sinquin, C, Fisher, AM & Boisson-Vidal, C, 1999, 'Further data on the structure of brown seaweed fucans: relationships with anticoagulant activity', *Carbohydr Res*, vol. 319, no. 1-4, pp. 154-65.

Chevolot, L, Mulloy, B, Ratiskol, J, Foucault, A & Colliec-Jouault, S, 2001, 'A disaccharide repeat unit is the major structure in fucoidans from two species of brown algae', *Carbohydr Res*, vol. 330, no. 4, pp. 529-35.

Chizhov, AO, Dell, A, Morris, HR, Haslam, SM, McDowell, RA, Shashkov, AS, Nifant'ev, NE, Khatuntseva, EA & Usov, AI, 1999, 'A study of fucoidan from the brown seaweed *Chorda filum'*, *Carbohydr Res*, vol. 320, no. 1-2, pp. 108-19.

Clementi, F, 1997, 'Alginate Production by *Azotobacter vinelandii'*, *Critical Reviews in Biotechnology*, vol. 17, no. 4, pp. 327-61.

Colin, S, Deniaud, E, Jam, M, Descamps, V, Chevolot, Y, Kervarec, N, Yvin, JC, Barbeyron, T, Michel, G & Kloareg, B, 2006, 'Cloning and biochemical characterization of the fucanase FcnA: definition of a novel glycoside hydrolase family specific for sulfated fucans', *Glycobiology*, vol. 16, no. 11, pp. 1021-32.

Conti, E, Flaibani, A, Regan, M & Sutherland, IW, 1994, 'Alginate from Pseudomonas fluorescens and P. putida: production and properties', *Microbiology*, vol. 140, no. 5, pp. 1125-32.

Cui, X, & Boland, T, 2009, 'Human microvasculature fabrication using thermal inkjet printing technology', *Biomaterials*, vol. 30, no. 31, pp. 6221-6227.

Daniel, R, Berteau, O, Chevolot, L, Varenne, A, Gareil, P & Goasdoue, N, 2001, 'Regioselective desulfation of sulfated L-fucopyranoside by a new sulfoesterase from the marine mollusk *Pecten maximus:* application to the structural study of algal fucoidan (*Ascophyllum nodosum*)', *Eur J Biochem*, vol. 268, no. 21, pp. 5617-26.

Datta, S, Barua, R & Das, J, 2019, 'Importance of Alginate Bioink for 3D Bioprinting in Tissue Engineering and Regenerative Medicine', in *Alginates-Recent Uses of This Natural Polymer*, IntechOpen.

Descamps, V, Colin, S, Lahaye, M, Jam, M, Richard, C, Potin, P, Barbeyron, T, Yvin, JC & Kloareg, B, 2006, 'Isolation and culture of a marine bacterium degrading the sulfated fucans from marine brown algae', *Marine Biotechnology (NY)*, vol. 8, no. 1, pp. 27-39.

Dou, W, Wei, D, Li, H, Li, H, Rahman, MM, Shi, J, Xu, Z & Ma, Y, 2013, 'Purification and characterisation of a bifunctional alginate lyase from novel *Isoptericola halotolerans* CGMCC 5336', *Carbohydr Polym*, vol. 98, no. 2, pp. 1476-82.

Doyle, JJ & Doyle, JL, 1987, *A rapid DNA isolation procedure for small quantities of fresh leaf tissue,* Phytochem Bull.

Duan, G, Han, F & Yu, W, 2009, 'Cloning, sequence analysis, and expression of gene alyPI encoding an alginate lyase from marine bacterium *Pseudoalteromonas* sp. CY24', *Can J Microbiol*, vol. 55, no. 9, pp. 1113-8.

Dumorné, K & Severe, R, 2018, 'Marine enzymes and their industrial and biotechnological applications', *Minerva Biotecnologica*, vol. 30, pp. 113-9.

Evans, JA, 2001, 'Molecular approaches to the isolation of rare actinomycete genera from Australia soils', Doctoral thesis, Department of Microbiology & Infectious Diseases, Flinders University.

Friedman, AJ, Phan, J, Schairer, DO, Champer, J, Qin, M, Pirouz, A, Blecher-Paz, K, Oren, A, Liu, PT, Modlin, RL & Kim, J, 2013, 'Antimicrobial and anti-inflammatory activity of chitosan-alginate nanoparticles: a targeted therapy for cutaneous pathogens', *J Invest Dermatol*, vol. 133, no. 5, pp. 1231-9.

Freier, T, Koh, HS, Kazazian, K, Shoichet, MS, 2005, 'Controlling cell adhesion and degradation of chitosan films by N-acetylation', *Biomaterials*, vol. 26, no. 29, pp. 5872-5878.

Furukawa, S, Fujikawa, T, Koga, D & Ide, A, 1992, 'Purification and Some Properties of Exo-type Fucoidanases from *Vibrio* sp. N-5', *Bioscience, Biotechnology, and Biochemistry*, vol. 56, no. 11, pp. 1829-34.

Gacesa, P & Wusteman, FS, 1990, 'Plate assay for simultaneous detection of alginate lyases and determination of substrate specificity', *Appl Environ Microbiol*, vol. 56, no. 7, pp. 2265-7.

Genilloud, O, 2017, 'Actinomycetes: still a source of novel antibiotics', *Natural product reports*, vol. 34, no. 10, pp. 1203-32.

Goodfellow, M & Williams, ST, 1983, 'Ecology of actinomycetes', *Annu Rev Microbiol*, vol. 37, pp. 189-216.

Granholm, V, 2014, 'The accuracy of statistical confidence estimates in shotgun proteomics', Doctoral thesis, comprehensive summary thesis, Department of Biochemistry and Biophysics, Stockholm University.

Gräslund, S, Nordlund, P, Weigelt, J, Hallberg, BM, Bray, J, Gileadi, O, Knapp, S, Oppermann, U, Arrowsmith, C, Hui, R, Ming, J, dhe-Paganon, S, Park, H-w, Savchenko, A, Yee, A, Edwards, A, Vincentelli, R, Cambillau, C, Kim, R, Kim, S-H, Rao, Z, Shi, Y, Terwilliger, TC, Kim, C-Y, Hung, L-W, Waldo, GS, Peleg, Y, Albeck, S, Unger, T, Dym, O, Prilusky, J, Sussman, JL, Stevens, RC, Lesley, SA, Wilson, IA, Joachimiak, A, Collart, F, Dementieva, I, Donnelly, MI, Eschenfeldt, WH, Kim, Y, Stols, L, Wu, R, Zhou, M, Burley, SK, Emtage, JS, Sauder, JM, Thompson, D, Bain, K, Luz, J, Gheyi, T, Zhang, F, Atwell, S, Almo, SC, Bonanno, JB, Fiser, A, Swaminathan, S, Studier, FW, Chance, MR, Sali, A, Acton, TB, Xiao, R, Zhao, L, Ma, LC, Hunt, JF, Tong, L, Cunningham, K, Inouye, M, Anderson, S, Janjua, H, Shastry, R, Ho, CK, Wang, D, Wang, H, Jiang, M, Montelione, GT, Stuart, DI, Owens, RJ, Daenke, S, Schütz, A, Heinemann, U, Yokoyama, S, Büssow, K, Gunsalus, KC, Structural Genomics, C, Architecture et Fonction des Macromolécules, B, Berkeley Structural Genomics, C, China Structural Genomics, C, Integrated Center for, S, Function, I, Israel Structural Proteomics, C, Joint Center for Structural, G, Midwest Center for Structural, G, New York Structural Genomi, XRCfSG, Northeast Structural Genomics, C, Oxford Protein Production, F, Protein Sample Production Facility, MDCfMM, Initiative, RSGP & Complexes, S, 2008, 'Protein production and purification', *Nature methods*, vol. 5, no. 2, pp. 135-46.

Gudapati, H, Yan, J, Huang, Y, Chrisey, DB, 2014, 'Alginate gelation-induced cell death during laser-assisted cell printing', *Biofabrication*,vol. 6, no. 3, pp. 022-035.

Gulve, R & Deshmukh, A, 2012, 'Antimicrobial activity of the marine actinomycetes', *International Multidisciplinary Research Journal*, vol. 2, no. 3.

Hartley, JL, 2006, 'Cloning technologies for protein expression and purification', *Current Opinion in Biotechnology*, vol. 17, no. 4, pp. 359-66.

Haug, A, Larsen, B & Smidsrod, O, 1967, 'Studies on the sequence of uronic acid residues in alginic acid', *Acta Chem Scand*, vol. 21, pp. 691-704.

Hoek, C, Mann, DG & Jahns, H, 1995, *Algea: An Introsuction to Phycology*, Cambridge University Press.

Hogue, M, Khaman, M & Shiekh, MA, 2006, 'Characterization and optimization of α-amylase activity of *Streptomyces clavifer*', *Pak. J. Biol. Sci*, vol. 9, pp. 1328-32.

Holdt, S & Kraan, S, 2011, 'Bioactive compounds in seaweed: functional food applications and legislation', *Journal of Applied Phycology*, vol. 23, no. 3, pp. 543-97.

Holtkamp, AD, Kelly, S, Ulber, R & Lang, S, 2008, 'Fucoidans and fucoidanases—focus on techniques for molecular structure elucidation and modification of marine polysaccharides', *Applied Microbiology and Biotechnology*, vol. 82, no. 1, pp. 1-11.

Holtzhauer, M, 2006, *Basic Methods for the Biochemical Lab*, Springer-Verlag Berlin Heidelberg, Berlin, Germany.

Inoue, A, 2018, 'Characterization of PL-7 family alginate lyases from marine organisms and their applications', *Methods Enzymol*, vol. 605, pp. 499-524.

Iskenderoglu, C, Acarturk, F, Erdogan, D & Bardakci, Y, 2013, 'In vitro and in vivo investigation of low molecular weight heparin-alginate beads for oral administration', *J Drug Target*, vol. 21, no. 4, pp. 389-406.

Islan, GA, Bosio, VE & Castro, GR, 2013, 'Alginate lyase and ciprofloxacin co-immobilization on biopolymeric microspheres for cystic fibrosis treatment', *Macromol Biosci*, vol. 13, no. 9, pp. 1238-48.

Islan, GA, Martinez, YN, Illanes, A & Castro, GR, 2014, 'Development of novel alginate lyase cross-linked aggregates for the oral treatment of cystic fibrosis', *RSC Advances*, vol. 4, no. 23, pp. 11758-65.

Jack, AA, Nordli, HR, Powell, LC, Farnell, DJJ, Pukstad, B, Rye, PD, Thomas, DW, Chinga-Carrasco, G & Hill, KE, 2019, 'Cellulose nanofibril formulations Incorporating a low-molecular-weight alginate oligosaccharide modify bacterial biofilm development', *Biomacromolecules*, vol. 20, no. 8, pp. 2953-61.

Jambeck, JR, Geyer, R, Wilcox, C, Siegler, TR, Perryman, M, Andrady, A, Narayan, R & Law, KL, 2015, 'Plastic waste inputs from land into the ocean', *Science*, vol. 347, no. 6223, pp. 768-71.

Jana, S & Deb, JK, 2005, 'Retracted article: Strategies for efficient production of heterologous proteins in *Escherichia coli*', *Applied Microbiology and Biotechnology*, vol. 67, no. 3, pp. 289-98.

Joo, GJ, 2005, 'Purification and characterization of an extracellular chitinase from the antifungal biocontrol agent *Streptomyces halstedii*', *Biotechnology letters*, vol. 27, no. 19, pp. 1483-6.

Kafilzadeh, F, Dehdari, F, Kadivar, E & Shiraz, OB, 2012, 'Isolation of amylase producing aquatic actinomycetes from the sediments of mangrove forests in south of Iran', *African Journal of Microbiology Research*, vol. 6, no. 33, pp. 6281-5.

Karlsson, E & Hirsh, I, 2011, 'Ion-Exchange Chromatography', in J-C Janson (ed.), *Protein Purification: Principles, High Resolution Methods, and Applications*, 3rd edn, John Wiley & Sons, Inc, New Jersey, USA, pp. 93-134.

Kawamoto, H, Horibe, A, Miki, Y, Kimura, T, Tanaka, K, Nakagawa, T, Kawamukai, M & Matsuda, H, 2006, 'Cloning and sequencing analysis of alginate lyase genes from the marine bacterium *Vibrio* sp. O2', *Mar Biotechnol (NY)*, vol. 8, no. 5, pp. 481-90.

Kelishomi, ZH, Goliaei, B, Mahdavi, H, Nikoofar, A, Rahimi, M, Moosavi-Movahedi, AA, Mamashli, F & Bigdeli, B, 2016, 'Antioxidant activity of low molecular weight alginate produced by thermal treatment', *Food Chem*, vol. 196, pp. 897-902.

Khalifa, S, Elias, N, Farag, MA, Chen, L, Saeed, A, Hegazy, M, Moustafa, MS, Wahed, A, Al-Mousawi, SM, Musharraf, SG, Chang, FR, Iwasaki, A, Suenaga, K, Alajlani, M, Göransson, U & El-Seedi, HR, 2019, 'Marine natural products: A source of novel anticancer drugs', *Mar Drugs*, vol. 17, no. 9, p. 491.

Khan, KH, 2013, 'Gene expression in mammalian cells and its applications', *Advanced pharmaceutical bulletin*, vol. 3, no. 2, pp. 257-63.

Khan, S, Ullah, MW, Siddique, R, Nabi, G, Manan, S, Yousaf, M & Hou, H, 2016, 'Role of recombinant DNA technology to improve life', *International journal of genomics*, vol. 2016, pp. 2405954-.

Kim, DE, Lee, EY & Kim, HS, 2009, 'Cloning and characterization of alginate lyase from a marine bacterium *Streptomyces* sp. ALG-5', *Mar Biotechnol (NY)*, vol. 11, no. 1, pp. 10-6.

Kim, EJ, Fathoni, A, Jeong, GT, Jeong, HD, Nam, TJ, Kong, IS & Kim, JK, 2013, 'Microbacterium oxydans, a novel alginate- and laminarin-degrading bacterium for the reutilization of brown-seaweed waste', *Journal of Environmental Management*, vol. 130, no. 0, pp. 153-9.

Kim, H, Lee, C & Lee, E, 2011, 'Alginate lyase: Structure, property, and application', *Biotechnology and Bioprocess Engineering*, vol. 16, no. 5, pp. 843-51.

Kim, HS, 2010, 'Characterization of Recombinant PolyG-Specific Lyase from a Marine Bacterium, *Streptomyces* sp. M3', *Journal of Life Science*, vol. - 20, no. 11, pp. 1582-8.

Kim, HT, Ko, HJ, Kim, N, Kim, D, Lee, D, Choi, IG, Woo, HC, Kim, MD & Kim, KH, 2012, 'Characterization of a recombinant endo-type alginate lyase (Alg7D) from *Saccharophagus degradans*', *Biotechnology Letters*, vol. 34, no. 6, pp. 1087-92.

Kitamikado, M, Tseng, CH, Yamaguchi, K & Nakamura, T, 1992, 'Two types of bacterial alginate lyases', *Appl Environ Microbiol*, vol. 58, no. 8, pp. 2474-8.

Kitamura, K, Matsuo, M & Tsuneo, Y, 1992, 'Enzymic Degradation of Fucoidan by Fucoidanase from the Hepatopancreas of *Patinopecten yessoensis'*, *Biosci Biotechnol Biochem*, vol. 56, no. 3, pp. 490-4.

Krishnakumar, S, Alexis Rajan, R & Ravikumar, S, 2011, 'Extracellular production of L-Glutaminase by marine alkalophilic *Streptomyces* sp.-SBU1 isolated from Cape Comorin coast', *Indian Journal of Marine Sciences*, vol. 40, no. 5, p. 717.

Kumar, L, Brice, J, Toberer, L, Klein-Seetharaman, J, Knauss, D & Sarkar, SK, 2019, 'Antimicrobial biopolymer formation from sodium alginate and algae extract using aminoglycosides', *PLoS One*, vol. 14, no. 3, p. e0214411.

Kumar, V, Bharti, A, Negi, YK, Gusain, O, Pandey, P & Bisht, GS, 2012, 'Screening of actinomycetes from earthworm castings for their antimicrobial activity and industrial enzymes', *Braz J Microbiol*, vol. 43, no. 1, pp. 205-14.

Kusaykin, Bakunina, I, Sova, V, Ermakova, S, Kuznetsova, T, Besednova, N, Zaporozhets, T & Zvyagintseva, T, 2008, 'Structure, biological activity, and enzymatic transformation of fucoidans from the brown seaweeds', *Biotechnol J*, vol. 3, no. 7, pp. 904-15.

Kusaykin, Chizhov, AO, Grachev, AA, Alekseeva, SA, Bakunina, IY, Nedashkovskaya, OI, Sova, VV & Zvyagintseva, TN, 2007, 'A comparative study of specificity of fucoidanases from marine microorganisms and invertebrates', in R Anderson, J Brodie, E Onsøyen & A Critchley (eds), *Eighteenth International Seaweed Symposium*, Springer Netherlands, vol. 1, pp. 143-7.

Kusaykin, M, Bakunina, I, Sova, V, Ermakova, S, Kuznetsova, T, Besednova, N, Zaporozhets, T & Zvyagintseva, T, 2008, 'Structure, biological activity, and enzymatic transformation of fucoidans from the brown seaweeds', *Biotechnology journal*, vol. 3, no. 7, pp. 904-15.

Kusaykin, MI, Silchenko, AS, Zakharenko, AM & Zvyagintseva, TN, 2016, 'Fucoidanases', *Glycobiology*, vol. 26, no. 1, pp. 3-12.

Kylin, H, 1913, *Zur Biochemie der Meeresalgen*, 3, Hoppe-Seyler´s Zeitschrift für physiologische Chemie.

Le, XH, Franco, CMM, Ballard, RA & Drew, EA, 2016, 'Isolation and characterisation of endophytic actinobacteria and their effect on the early growth and nodulation of lucerne (Medicago sativa L.)', *Plant and Soil*, vol. 405, no. 1, pp. 13-24.

Lee, B, 2008, *Seaweed potential as a marine vegetable and other opportunities* RIRDC Publication No 08/009.

Lee, B, 2010, *Cultivated seaweed and seaweed industry development in Australia*, RIRDC publication No. 10/164.

Lee, KY & Mooney, DJ, 2012, 'Alginate: Properties and biomedical applications', *Progress in Polymer Science*, vol. 37, no. 1, pp. 106-26.

LeRoux, MA, Guilak, F & Setton, LA, 1999, 'Compressive and shear properties of alginate gel: effects of sodium ions and alginate concentration', *J Biomed Mater Res*, vol. 47, no. 1, pp. 46-53.

Lewis, JR, 1964, *The Ecology of Rocky Shores* The English Universities Press Ltd.

Li, S, Wang, Y, Li, X, Lee, BS, Jung, S & Lee, MS, 2019, 'Enhancing the thermo-stability and anti-biofilm activity of alginate lyase by immobilization on low molecular weight chitosan nanoparticles', *Int J Mol Sci*, vol. 20, no. 18.

Li, S, Yang, X, Zhang, L, Yu, W & Han, F, 2015, 'Cloning, expression, and characterization of a cold-adapted and surfactant-stable alginate lyase from marine bacterium *Agarivorans* sp. L11', *J Microbiol Biotechnol*, vol. 25, no. 5, pp. 681-6.

Liu, Q, Li, Q, Xu, S, Zheng, Q & Cao, X, 2018, 'Preparation and properties of 3D printed alginate–chitosan polyion complex cydrogels for tissue engineering', *Polymers*, vol. 10, no. 6, p. 664.

Lorbeer, A, Tham, R & Zhang, W, 2013, 'Potential products from the highly diverse and endemic macroalgae of Southern Australia and pathways for their sustainable production', *Journal of Applied Phycology*, vol. 25, no. 3, pp. 717-32.

Lorbeer, AJ, Charoensiddhi, S, Lahnstein, J, Lars, C, Franco, CMM, Bulone, V & Zhang, W, 2017, 'Sequential extraction and characterization of fucoidans and alginates from *Ecklonia radiata, Macrocystis pyrifera, Durvillaea potatorum,* and *Seirococcus axillaris'*, *Journal of Applied Phycology*, vol. 29, no. 3, pp. 1515-26.

Madgwick, J, Haug, A & Larsen, B, 1978, 'Ionic requirements of alginate-modifying enzymes in the marine alga *Pelvetia canaliculata* (L.) Dcne. et Thur.', *Botanica Marina*, vol. 21, no. 1, pp. 1-4.

Maleki, H & Mashinchian, O, 2011, 'Characterization of *Streptomyces* ssolates with UV, FTIR spectroscopy and HPLC analyses', *BioImpacts : BI*, vol. 1, no. 1, pp. 47-52.

Malissard, M, Chavagnat, F, Duez, C, Vacheron, MJ, Guinand, M, Michel, G & Ghuysen, JM, 1995, 'Overproduction and properties of the mannuronate alginate lyase AlxMB', *FEMS Microbiol Lett*, vol. 126, no. 2, pp. 105-11.

Malissard, M, Duez, C, Guinand, M, Vacheron, MJ, Michel, G, Marty, N, Joris, B, Thamm, I & Ghuysen, JM, 1993, 'Sequence of a gene encoding a (poly ManA) alginate lyase active on *Pseudomonas aeruginosa* alginate', *FEMS Microbiol Lett*, vol. 110, no. 1, pp. 101-6.

Manilal, A, Sujith, S, Kiran, GS, Selvin, J, Shakir, C, Gandhimathi, R & Panikkar, MN, 2009, 'Biopotentials of seaweeds collected from southwest coast of India', *Journal of Marine Science and Technology*, vol. 17, no. 1, pp. 67-73.

Manivasagan, P & Oh, J, 2015, 'Production of a Novel Fucoidanase for the Green Synthesis of Gold Nanoparticles by *Streptomyces* sp. and Its Cytotoxic Effect on HeLa Cells', *Marine Drugs*, vol. 13, no. 11, pp. 6818-37.

Miller, GL, 1959, 'Use of dinitrosalicylic acid reagent for determination of reducing sugar', *Analytical Chemistry*, vol. 31, no. 3, pp. 426-8.

Nandakumar, R, Yoshimune, K, Wakayama, M & Moriguchi, M, 2003, 'Microbial glutaminase: biochemistry, molecular approaches and applications in the food industry', *Journal of Molecular Catalysis B: Enzymatic*, vol. 23, no. 2–6, pp. 87-100.

Narayana, KJ & Vijayalakshmi, M, 2009, 'Chitinase production by *Streptomyces* sp. ANU 6277', *Brazilian Journal of Microbiology*, vol. 40, no. 4, pp. 725-33.

Nawani, N, Aigle, B, Mandal, A, Bodas, M, Ghorbel, S & Prakash, D, 2013, 'Actinomycetes: role in biotechnology and medicine', *BioMed research international*, vol. 2013, pp. 6871-90.

Nishino, T, Nagumo, T, Kiyohara, H & Yamada, H, 1991, 'Structural characterization of a new anticoagulant fucan sulfate from the brown seaweed *Ecklonia kurome*', *Carbohydr Res*, vol. 211, no. 1, pp. 77-90.

Nisizawa, K, Fujibayashi, S & Kashiwabara, Y, 1968, 'Alginate lyases in the hepatopancreas of a marine mollusc, *Dolabella auricula* Solander', *J Biochem*, vol. 64, no. 1, pp. 25-37.

Ochiai, A, Yamasaki, M, Mikami, B, Hashimoto, W & Murata, K, 2010, 'Crystal structure of exotype alginate lyase Atu3025 from *Agrobacterium tumefaciens*', *J Biol Chem*, vol. 285, no. 32, pp. 24519-28.

Oussalah, M, Caillet, S, Salmieri, S, Saucier, L & Lacroix, M, 2006, 'Antimicrobial effects of alginate-based film containing essential oils for the preservation of whole beef muscle', *J Food Prot*, vol. 69, no. 10, pp. 2364-9.

Osawa, T, Matsubara, Y, Muramatsu, T, Kimura, M & Kakuta, Y, 2005, 'Crystal structure of the alginate (poly alpha-l-guluronate) lyase from *Corynebacterium* sp. at 1.2 A resolution', *J Mol Biol*, vol. 345, no. 5, pp. 1111-8.

Park, D, Jagtap, S & Nair, SK, 2014, 'Structure of a PL17 family alginate lyase demonstrates functional similarities among exotype depolymerases', *J Biol Chem*, vol. 289, no. 12, pp. 8645-55.

Park, DH, Kim, JS, Kwon, SW, C, Yu, YM, Hur, JH, Lim, CK, 2003, '*Streptomyces luridiscabiei* sp. nov., *Streptomyces puniciscabiei* sp. nov. and *Streptomyces niveiscabiei* sp. nov., which cause potato common scab disease in Korea', *Int J Syst Evol Microbiol*, vol 53, pp 2049-54.

Park, HH, Kam, N, Lee, EY & Kim, HS, 2012, 'Cloning and characterization of a novel oligoalginate lyase from a newly isolated bacterium *Sphingomonas* sp. MJ-3', *Mar Biotechnol (NY)*, vol. 14, no. 2, pp. 189-202.

Park, HJ, Ahn, JM, Park, RM, Lee, SH, Sekhon, SS, Kim, SY, Wee, JH, Kim, YH & Min, J, 2016, 'Effects of Alginate Oligosaccharide Mixture on the Bioavailability of

Lysozyme as an Antimicrobial Agent', *J Nanosci Nanotechnol*, vol. 16, no. 2, pp. 1445-9.

Parker, L, 2018, *Here's how much plastic trash is littering the earth*, National Geographic.

Patel, KK, Tripathi, M, Pandey, N, Agrawal, AK, Gade, S, Anjum, MM, Tilak, R & Singh, S, 2019, 'Alginate lyase immobilized chitosan nanoparticles of ciprofloxacin for the improved antimicrobial activity against the biofilm associated mucoid P. aeruginosa infection in cystic fibrosis', *Int J Pharm*, vol. 563, pp. 30-42.

Pawar, SN & Edgar, KJ, 2012, 'Alginate derivatization: A review of chemistry, properties and applications', *Biomaterials*, vol. 33, no. 11, pp. 3279-305.

Pecina, A & Paneque, A, 1994, 'Studies on some enzymes of alginic acid biosynthesis in mucoid and nonmucoid *Azotobacter chroococcum* strains', *Applied Biochemistry and Biotechnology*, vol. 49, no. 1, pp. 51-8.

Phillips, JA, 2001, 'Marine macroalgal biodiversity hotspots: why is there high species richness and endemism in southern Australian marine benthic flora?', *Biodiversity & Conservation*, vol. 10, no. 9, pp. 1555-77.

Pio, TF & Macedo, GA, 2008, 'Cutinase production by Fusarium oxysporum in liquid medium using central composite design', *J Ind Microbiol Biotechnol*, vol. 35, no. 1, pp. 59-67.

Preston, JF, Romeo, T, Bromley, JC, Robinson, RW & Aldrich, HC, 1985, ' Alginate lyase-secreting bacteria associated with the algal genus *Sargassum*', *Dev. Ind. Microbiology*, no. 26, pp. 727 - 40.

Qianqian, W, Shuang, M, Hourong, X, Min, Z & Jingmin, C, 2011, 'Purification and the Secondary Structure of Fucoidanase from *Fusarium* sp. LD8', *Evid Based Complement Alternat Med*, vol. 2011, p. 196190.

Ragunathan, R & Padhmadas, R, 2013, 'Production, purification and characterization of α-amylase using *Streptomyces* spp. PDS1 and *Rhodococcu*s spp. Isolatedfrom Western Ghats', *International Journal of Current Microbiology and Applied Sciences*, vol. 2, no. 8.

Rahman, MM, Inoue, A, Tanaka, H & Ojima, T, 2010, 'Isolation and characterization of two alginate lyase isozymes, AkAly28 and AkAly33, from the common sea hare *Aplysia kurodai*', *Comp Biochem Physiol B Biochem Mol Biol*, vol. 157, no. 4, pp. 317-25.

Ramesh, S & Mathivanan, N, 2009, 'Screening of marine actinomycetes isolated from the Bay of Bengal, India for antimicrobial activity and industrial enzymes', *World Journal of Microbiology and Biotechnology*, vol. 25, no. 12, pp. 2103-11.

Reda, FM, 2015, 'Kinetic properties of *Streptomyces canarius* L- Glutaminase and its anticancer efficiency', *Brazilian Journal of Microbiology*, vol. 46, pp. 957-68.

Rioux, LE, Turgeon, SL & Beaulieu, M, 2007, 'Characterization of polysaccharides extracted from brown seaweeds', *Carbohydr Polym*, vol. 69, no. 3, pp. 530-7.

Rizkia, PR, Silaban, S, Hasan, K, Kamara, DS, Subroto, T, Soemitro, S & Maksum, IP, 2015, 'Effect of isopropyl-β-D-thiogalactopyranoside concentration on prethrombin-2 recombinan gene expression in *Escherichia Coli* ER2566', *Procedia Chemistry*, vol. 17, pp. 118-24.

Rodriguez-Jasso, RM, Mussatto, SI, Pastrana, L, Aguilar, CN & Teixeira, JA, 2010, 'Fucoidan-degrading fungal strains: screening, morphometric evaluation, and influence of medium composition', *Appl Biochem Biotechnol*, vol. 162, no. 8, pp. 2177-88.

Rosano, GL & Ceccarelli, EA, 2014, 'Recombinant protein expression in *Escherichia coli*: advances and challenges', *Front Microbiol*, vol. 5, p. 172.

Sakai, T, Kawai, T & Kato, I, 2004, 'Isolation and characterization of a fucoidan-degrading marine bacterial strain and its fucoidanase', *Mar Biotechnol (NY)*, vol. 6, no. 4, pp. 335-46.

Sakatoku, A, Wakabayashi, M, Tanaka, Y, Tanaka, D & Nakamura, S, 2012, 'Isolation of a novel *Saccharophagus species* (Myt-1) capable of degrading a variety of seaweeds and polysaccharides', *MicrobiologyOpen*, vol. 1, no. 1, pp. 2-12.

Salami, F, 2004, 'Isolation and determination of *Streptomyces* that produce antibiotic from soil', *Pajouhesh-va-sazandegi*.

Salem, D, Sallam, MAE & Youssef, T, 2019, 'Synthesis of compounds having antimicrobial activity from alginate', *Bioorg Chem*, vol. 87, pp. 103-11.

Santos, SA, Vilela, C, Freire, CS, Abreu, MH, Rocha, SM & Silvestre, AJ, 2015, '*Chlorophyta* and *Rhodophyta* macroalgae: a source of health promoting phytochemicals', *Food Chem*, vol. 183, pp. 122-8.

Sarada, KV, 2013, 'Production and applications of L-Glutaminase using fermentation technology', *Asia Pacific Journal of Research*, vol. 1, no. 8.

Sasaki, K, Sakai, T, Kojima, K, Nakayama, S, Nakanishi, Y & Kato, I, 1996, 'Partial purification and characterization of an enzyme releasing 2-sulfo-alpha-L-fucopyranose from 2-sulfo-alpha-L-fucopyranosyl-(1->2) pyridylaminated fucose from a sea urchin, *Strongylocentrotus nudus*', *Biosci Biotechnol Biochem*, no. 60, pp. 666 - 8.

Sawabe, T, Ezura, Y & Kimura, T, 1992, 'Purification and Characterization of an Alginate Lyase from Marine *Alteromonas* sp', *Nippon Suisan Gakkaishi*, vol. 58, no. 3, pp. 521-7.

Sawabe, T, Ohtsuka, M & Ezura, Y, 1997, 'Novel alginate lyases from marine bacterium *Alteromonas* sp. strain H-4', *Carbohydr Res*, vol. 304, no. 1, pp. 69-76.

Sawant, SS, Salunke, BK & Kim, BS, 2015, 'A rapid, sensitive, simple plate assay for detection of microbial alginate lyase activity', *Enzyme Microb Technol*, vol. 77, pp. 8-13.

Schaumann, K & Weide, G, 1990, 'Enzymatic degradation of alginate by marine fungi', *Hydrobiologia*, vol. 204, no. 1, pp. 589-96.

Schlieker, C, Bukau, B & Mogk, A, 2002, 'Prevention and reversion of protein aggregation by molecular chaperones in the *E. coli* cytosol: implications for their applicability in biotechnology', *J Biotechnol*, vol. 96, no. 1, pp. 13-21.

Schneider, B, 2010, *Nuclear Magnetic Resonance Spectroscopic Analysis of Enzyme Products - Progress in Botany 72*, vol. 72, Springer, Berlin, Heidelberg.

Selvam, K, 2011, 'Screening and quantification of marine actinomycetes producing industrial enzymes amylase, cellulase and lipase from south coast of India', *International Journal of Pharmaceutical & Biological Archive*, vol. 2, no. 5.

Sen, F, Uzunsoy, I, Basturk, E & Kahraman, MV, 2017, 'Antimicrobial agent-free hybrid cationic starch/sodium alginate polyelectrolyte films for food packaging materials', *Carbohydr Polym*, vol. 170, pp. 264-70.

Sen, M, 2011, 'Effects of molecular weight and ratio of guluronic acid to mannuronic acid on the antioxidant properties of sodium alginate fractions prepared by radiation-induced degradation', *Appl Radiat Isot*, vol. 69, no. 1, pp. 126-9.

Shafiei, M, Ziaee, AA & Amoozegar, MA, 2011, 'Purification and characterization of an organic-solvent-tolerant halophilic α-amylase from the moderately halophilic *Nesterenkonia* sp. strain F', *J Ind Microbiol Biotechnol*, vol. 38, no. 2, pp. 275-81.

Shankar, S & Rhim, JW, 2018, 'Antimicrobial wrapping paper coated with a ternary blend of carbohydrates (alginate, carboxymethyl cellulose, carrageenan) and grapefruit seed extract', *Carbohydr Polym*, vol. 196, pp. 92-101.

Shimokawa, T, Yoshida, S, Takeuchi, T, Murata, K, Kobayashi, H & Kusakabe, I, 1997, 'Purification and characterization of extracellular poly(-D-1,4-mannuronide) lyase from *Dendryphiella salina* IFO 32139', *Biosci. Biotechnol. Biochem.*, no. 61, pp. 636 - 40.

Shin, JW, Choi, SH, Kim, DE, Kim, HS, Lee, JH, Lee, IS & Lee, EY, 2011, 'Heterologous expression of an alginate lyase from *Streptomyces* sp. ALG-5 in *Escherichia coli* and its use for preparation of the magnetic nanoparticle-immobilized enzymes', *Bioprocess Biosyst Eng*, vol. 34, no. 1, pp. 113-9.

Shiraiwa, Y, Abe, K, Sasaki, SF, Ikawa, T & Nisizawa, K, 1975, 'Alginate lyase activities in the extracts from several brown algae', *Botanica Marina*, vol. 18, no. 2, pp. 97 - 104.

Silaban, S, Gaffar, S, Simorangkir, M, Maksum, IP & Subroto, T, 2019, 'Effect of IPTG concentration on recombinant human prethrombin-2 expression in *Escherichia coli* BL21(DE3) ArcticExpress', *IOP Conference Series: Earth and Environmental Science*, vol. 217, p. 012039.

Silchenko, AS, Kusaykin, MI, Kurilenko, VV, Zakharenko, AM, Isakov, VV, Zaporozhets, TS, Gazha, AK & Zvyagintseva, TN, 2013, 'Hydrolysis of fucoidan by fucoidanase isolated from the marine bacterium, *Formosa algae*', *Mar Drugs*, vol. 11, no. 7, pp. 2413-30.

Singh, D, Gupta, A, Wilkens, SL, Mathur, AS, Tuli, DK, Barrow, CJ & Puri, M, 2015, 'Understanding response surface optimisation to the modeling of Astaxanthin extraction from a novel strain *Thraustochytrium* sp. S7', *Algal Research*, vol. 11, pp. 113-20.

Song, MY, Ku, SK & Han, JS, 2012, 'Genotoxicity testing of low molecular weight fucoidan from brown seaweeds', *Food Chem Toxicol*, vol. 50, no. 3-4, pp. 790-6.

Storz, H, Zimmermann, U, Zimmermann, H & Kulicke, WM, 2010, 'Viscoelastic properties of ultra-high viscosity alginates', *Rheologica Acta*, vol. 49, no. 2, pp. 155-67.

Subramani, R & Sipkema, D, 2019, 'Marine rare actinomycetes: A promising source of structurally diverse and unique novel natural products', *Mar Drugs*, vol. 17, no. 5, p. 249.

Suda, K, Tanji, Y, Hori, K & Unno, H, 1999, 'Evidence for a novel *Chlorella* virus-encoded alginate lyase', *FEMS Microbiol Lett*, vol. 180, no. 1, pp. 45-53.

Suneetha, V & Khan, ZA, 2011, 'Actinomycetes: sources for soil enzymes', in *Soil Enzymology*, Springer, pp. 259-69.

Taechowisan, T, Peberdy, J & Lumyong, S, 2003, 'Chitinase production by endophytic *Streptomyces aureofaciens* CMUAc130 and its antagonism against phytopathogenic fungi', *Annals of microbiology*, vol. 53, no. 4, pp. 447-62.

Takayama, M, Koyama, N, Sakai, T & Kato, I, 2002, *Enzymes capable of degrading a sulfated-fucose-containing polysaccharide and their encoding genes*, Google Patents, <http://www.google.com/patents/US6489155>.

Tang, JC, Taniguchi, H, Chu, H, Zhou, Q & Nagata, S, 2009, 'Isolation and characterization of alginate-degrading bacteria for disposal of seaweed wastes', *Lett Appl Microbiol*, vol. 48, no. 1, pp. 38-43.

Tang, S, Wang, Z, Li, P, Li, W, Li, C, Wang, Y & Chu, PK, 2018, 'degradable and photocatalytic antibacterial Au-TiO(2)/sodium alginate nanocomposite films for active food packaging', *Nanomaterials (Basel)*, vol. 8, no. 11.

Thanassi, NM & Nakada, HI, 1967, 'Enzymatic dagradation of fucoidan by enzymes from the hepatopancreas of abalone *Haliotus* species', *Archives of Biochemistry and Biophysics*, vol. 1, no. 118, pp. 172 - 7.

Thomas, F, Lundqvist, LC, Jam, M, Jeudy, A, Barbeyron, T, Sandstrom, C, Michel, G & Czjzek, M, 2013, 'Comparative characterization of two marine alginate lyases from Zobellia galactanivorans reveals distinct modes of action and exquisite adaptation to their natural substrate', *J Biol Chem*, vol. 288, no. 32, pp. 23021-37.

Uchimura, K, Miyazaki, M, Nogi, Y, Kobayashi, T & Horikoshi, K, 2010, 'Cloning and sequencing of alginate lyase genes from deep-sea strains of *Vibrio* and *Agarivorans* and characterization of a new *Vibrio* enzyme', *Mar Biotechnol (NY)*, vol. 12, no. 5, pp. 526-33.

Vasseur, E, 1948, 'Chemical studies on the jelly coat of the sea-urchin egg', *Acta Chem. Scand*, vol. 2, pp. 900 - 13.

Vo, T, 2018, 'Actinobacteria endophyte as beneficial partners for chickpea production', Masters thesis, Department of Medical Biotechnology, Flinders University.

Wang, Y, Chen, X, Bi, X, Ren, Y, Han, Q, Zhou, Y, Han, Y, Yao, R & Li, S, 2019, 'Characterization of an alkaline alginate lyase with pH-stable and thermo-tolerance property', *Mar Drugs*, vol. 17, no. 5.

Wang, Y, Guo, EW, Yu, WG & Han, F, 2013, 'Purification and characterization of a new alginate lyase from a marine bacterium *Vibrio* sp', *Biotechnol Lett*, vol. 35, no. 5, pp. 703-8.

Wang, Y, Wang, Z, Duo, Y, Wang, X, Chen, J & Chen, J, 2018, 'Gene cloning, expression, and reducing property enhancement of nitrous oxide reductase from *Alcaligenes denitrificans* strain TB', no. 1873-6424 (Electronic).

Wasserman, BP, 1995, 'Principle of enzymology for the food sciences, 2nd ediction', *Journal of Food Safety*, vol. 15, no. 4, pp. 365a-6.

Wijesinghe, WA & Jeon, YJ, 2012, 'Enzyme-assistant extraction (EAE) of bioactive components: a useful approach for recovery of industrially important metabolites from seaweeds: a review', *Fitoterapia*, vol. 83, no. 1, pp. 6-12.

Wilson, K, 2010, 'Chromatographic techniques', in K Wilson & JM Walker (eds), *Principles and Techniques of Biochemistry and Molecular Biology*, 7th edn, Cambridge University Press, Cambridge, UK, pp. 433-76.

Wong, TY, Preston, LA & Schiller, NL, 2000, 'Alginate lyase: review of major sources and enzyme characteristics, structure-function analysis, biological roles, and applications', *Annu Rev Microbiol*, vol. 54, pp. 289-340.

Wright, F & Bibb, MJ, 1992, 'Codon usage in the G+C-rich *Streptomyces genome*', *Gene*, vol. 113, no. 1, pp. 55-65.

Wu, Q, Zhang, M, Wu, K, Liu, B, Cai, J & Pan, R, 2011, 'Purification and characteristics of fucoidanase obtained from *Dendryphiella arenaria* TM94', *Journal of Applied Phycology*, vol. 23, no. 2, pp. 197-203.

Yamasaki, M, Ogura, K, Hashimoto, W, Mikami, B & Murata, K, 2005, 'A structural basis for depolymerization of alginate by polysaccharide lyase Family-7', *Journal of Molecular Biology*, vol. 352, no. 1, pp. 11-21.

Yan, J, Chen, P, Zeng, Y, Men, Y, Mu, S, Zhu, Y, Chen, Y & Sun, Y, 2019, 'The characterization and modification of a novel bifunctional and robust alginate lyase derived from *Marinimicrobium* sp. H1', *Mar Drugs*, vol. 17, no. 10.

Yanez, M, Rincon, J, Dones, A, De Maria, C, Gonzales, R, Boland, T, 2015, 'In Vivo assessment of printed microvasculature in a bilayer skin graft to treat fullthickness wounds', *Tissue Engineering Part A*, vol. 2, no. 1, pp. 224-233.

Yang, M, Yu, Y, Yang, S, Shi, X, Mou, H & Li, L, 2018, 'Expression and characterization of a new PolyG-specific alginate lyase from marine bacterium *Microbulbifer* sp. Q7', *Front Microbiol*, vol. 9, no. 2894.

Yassien, MAM & Asfour, HZ, 2011, 'Production of α-amylase by *Streptomyces* species isolated from West Area of Saudi Arabia', *Journal of Pharmacy Research*, vol. 4, no. 12.

Yu, Z, Zhu, B, Wang, W, Tan, H & Yin, H, 2018, 'Characterization of a new oligoalginate lyase from marine bacterium *Vibrio* sp', *Int J Biol Macromol*, vol. 112, pp. 937-42.

Zellner, M, Winkler, W, Hayden, H, Diestinger, M, Eliasen, M, Gesslbauer, B, Miller, I, Chang, M, Kungl, A, Roth, E & Oehler, R, 2005, 'Quantitative validation of different protein precipitation methods in proteome analysis of blood platelets', *Electrophoresis*, vol. 26, no. 12, pp. 2481-9.

Zhang, C & Kim, S, 2010, 'Research and application of marine microbial enzymes: status and prospects', *Mar Drugs*, vol. 8, no. 6, pp. 1920-34.

Zhu, B, Sun, Y, Ni, F, Ning, L & Yao, Z, 2018, 'Characterization of a new endo-type alginate lyase from *Vibrio* sp. NJU-03', *International Journal of Biological Macromolecules*, vol. 108, pp. 1140-7.

Zhu, B & Yin, H, 2015, 'Alginate lyase: Review of major sources and classification, properties, structure-function analysis and applications', *Bioengineered*, vol. 6, no. 3, pp. 125-31.

Zhu, X, Li, X, Shi, H, Zhou, J, Tan, Z, Yuan, M, Yao, P & Liu, X, 2018, 'Characterization of a Novel Alginate Lyase from Marine Bacterium *Vibrio furnissii* H1', *Marine Drugs*, vol. 16, no. 1, p. 30.

Zhu, Y, Wu, L, Chen, Y, Ni, H, Xiao, A & Cai, H, 2016, 'Characterization of an extracellular biofunctional alginate lyase from marine *Microbulbife*r sp. ALW1 and antioxidant activity of enzymatic hydrolysates', *Microbiol Res*, vol. 182, pp. 49-58.

Zhu, Z, Cheng, X, Gao, D, Xu, P, Guo, Q, Sun, D, Qin, H-M & Lu, F, 2018, 'Research progress of alginate lyases on function and application', *IOP conference series.*, vol. 199, no. 5, p. 052016.