Bioprospecting of novel Thraustochytrids from Scotland for high value compounds

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ABSTRACT

Thraustochytrids are marine microheterotrophs and important producers of many industrially relevant compounds such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), extracellular enzymes, carotenoids, and proteins. Despite their growing importance in recent years, there is currently very limited data regarding the biodiversity and biotechnological potential of Scottish strains.

The project aimed to isolate novel thraustochytrids from a temperate climate of Scotland and to screen them for the production of biotechnologically relevant compounds. To achieve this, twenty thraustochytrids were isolated from the Scottish saltmarshes and screened for the presence of lipids, extracellular enzymes, total proteins, antimicrobial agents, and biosurfactants by the adoption of the sulfo-phospho colorimetric assay, substrate digestion plates, Bradford assay, disc diffusion assay, and emulsification assay, respectively.

The results showed a relative abundance of thraustochytrids in the Scottish coastal regions as well as established a reliable method of their isolation from this area. Moreover, the study confirmed the ability of thraustochytrids to produce many important hydrolyzing enzymes including chitinase, cellulase, and amylase. Isolated thraustochytrids were also able to synthesize emulsifying agents, an ability that has never been reported before. Overall, the results here show that isolated thraustochytrids produce a variety of biotechnologically relevant compounds with the potential to contribute to key biotechnological sectors.

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Chapter 1– INTRODUCTION

1.1 Taxonomy and morphological features of Thraustochytrids

1.1.1 Taxonomy

Since the first isolation of *Thraustochytrium proliferum* in 1936 from the coastal waters of Woods Hole, Massachusetts by Sparrow, many scientists have tried to establish true taxonomic position of thraustochytrids [1]. Initially, thraustochytrids were assigned to the order Saprolegniales (Phylum: Oomycota) due to their possession of biflagellate zoospores [2], [3]. However, as ultrastructural evidence revealed unique features of these organisms such as a monocentric thallus and ectoplasmic net system (EN), Alderman et al. (1974) [4] suggested establishing the separate order Thraustoehytriales to accommodate them within the Oomycota phylum. It wasn't until 18S rDNA sequencing technology became available that Cavalier-Smith et al., (1994) [5] stripped thraustochytrids of their direct affiliation with fungi and place them in the family *Thraustochytridae* and assigned to the class Labyrinthulea together with the family *Labyrinthulidae*. The class Labyrinthulea (also known as Labyrinthulomycetes) included thraustochytrids and other EN producers namely, aplanochytrids (represented by a single genus of *Aplanochytrium*) and the labyrinthulids. Furthermore, a 18S rDNA study by Honda et al., (1999) [6] reinforced these interrelations and demonstrated that thraustochytrids, aplanochytrids, and labyrinthulids formed a monophyletic grouping within the Stramenopile kingdom. This was in line with recent findings that confirmed that Labyrinthulomycetes, opalinids, and human parasite *Blastocystis* are in fact, part of one early-diverging brach of Stramenopiles and that Oomycetes form another distinct lineage despite having a common ancestor [7].

The current taxonomic consensus is largely based on the work of Dick (2001) [8] with two dominant families *Thraustochytriaceae* and *Labyrinthuaceae* (**Table 1.1**). Recent changes include the addition of two families the *Aplanochytriacae* and *Stellarchytriacae* to the order Labyrinthulida [7]. The family *Aplanochytriacae* includes some members of the *Thraustochytrium* genus that were in the *Thraustochytriacae* family whereas, the family *Stellarchytriacae* (containing only one genus *Stellarchytrium*) has been provisionally placed in Labyrinthulida and contains undescribed strains known only from environmental sequencing [9]. In terms of the order Thraustochytriales,

thraustochytrid-like *Oblongichytrium* genus was removed from the Thraustochytriaceae family, placing it into its own *Oblongichytriidae* family [9] (**Table 1.1**).

Table 1.1 Taxonomic classification of Labyrinthulomycetes. Adpoted from Archibald et al., (2017) [7].

Kingdom	**Straminipila (Heterokonta)**		
Phylum	Labyrinthulomycota		
Class	Labyrinthulomycete		
Order	Labyrinthulida	Oblongichytridiales	Thraustochytriales
Family	*Aplanochytriaceae* *Stellarchytriaceae** *Labyrinthulaceae*	*Oblongichytridiaceae*	*Althornidiaceae* *Thraustochytriacae*
Genus	*Aplanochytrium* *Stellarchytrium* *Labyrinthula*	*Oblongichytrium*	*Althornia* *Aurantiochytrium, Botryochytrium, Japanochytrium, Monorhizochytrium, Parietichytrium, Schizochytrium, Sicyoidochytrium, Thraustochytrium, Ulkenia*

* Provisional allocation [9]

1.1.2 Description of the genera

The order Thraustochytriales is the most diverse and the largest order within the class Labyrinthulomycete. These are mainly marine saprophytes and detritus dwellers where they use their fine EN to attach to the substrata [7]. At present, there are two families within the order Thraustochytriale, *Althornidiaceae* with a single genus *Althornia*, and *Thraustochytriacae* (commonly referred to as "Thraustochytrids"), containing nine genera: (*Aurantiochytrium, Botryochytrium, Japanochytrium, Monorhizochytrium, Parietichytrium, Schizochytrium, Sicyoidochytrium, Thraustochytrium,* and *Ulkenia* [7]). *Althornidiaceae* contains only one species (*Althornia crouchii)* which does not produce the usual EN but rather absorbs nutrients directly from the environment and constitutes a part of free-floating plankton community. Furthermore, *Althornidiaceae* is the only family to date which taxonomic allocation was based solely on morphology

with no sequence data available [7]. *Thraustochytriacae* is a large family with a morphologically diverse genera that possess a highly developed EN and was established as a result of multiple molecular studies [10]. Since *Althornidiaceae* has been provisionally placed within the Thraustochytriales, this review will focus only on the description of the family *Thraustochytriacae*.

1.1.3 Morphology and cell cycle

Thraustochytrids are diploid, uni-cellular, and non-motile, and typically, the cells are ovoid or globular in shape. In contrast to labyrinthulids, they do not use their EN for movement but rather to allow adhesion and penetration of solids surfaces and to absorb nutrients [11]. The cell wall of thraustochytrids is non-cellulosic and consists of sulfated polysaccharides enriched with xylose and galactose [12]. Thraustochytrids reproduce asexually by releasing highly motile zoospores and the mode of zoospore production has been used for genera differentiation before other criteria such as DHA accumulation or carotenoid production were introduced [10]. In brief, the cytoplasm of a vegetative cell either develops into zoosporangium and then divides into zoospores (*Thraustochytrium* genus) or abandons the cell to form a free-floating ameboidal mass before zoospore division (*Ulkenia* genus) [13]. Therefore, some thraustochytrids that contain the ameboid stage can play a dual role in the marine ecosystem. As a organic matter degrader when they are in their thallic form and as a motile, scavenging phagothroph in the ameboid form [14]. Another type of division in thraustochytrids is denoted by successive bi-partitioning of a thallus into diads, tetrads, and clusters before the development of zoosporangium and release of spores (*Schizochytrium* genus). Yokoyama et al., (2007) [10] expanded the family *Thraustochytriacae* further by adding new genera, namely: *Aurantiochytrium, Botryochytrium, Parietichytrium,* and *Sicyoidochytrium*. The addition was due to the differences in pigment production or the amount of DHA accumulation. According to recent reports, thraustochytrids may also undergo sexual reproduction by production and fusion of haploid gametes as per observations of Ganuza et al., (2019) [15] but more evidence is required to fully validate this claim.

1.1.3.1 Aurantiochytrium

The genus Aurantiochytrium contains three defined species *Aurantiochytrium limacinum* [6], *Aurantiochytrium mangrovei* [16], and most recent *Aurantiochytrium acetophilum* [15]. *Aurantiochytrium* is a relatively novel genus created by Yokoyama et Honda., (2007) [10] from *Schizochytrium* species that contained less than 5% of arachidonic acid, lacked EN, and produced a large amount of astaxanthin. Similar to *Schizochytrium*, *Aurantiochytrium* is an excellent DHA producer and has been extensively used in the biotechnological industry [17], [18], [19]. They contain a thin-walled thallus and various carotenoids like astaxanthin, canthaxanthin and β-carotene which pigment their colonies orange. In addition, most of them divide by continuous binary cell divisions with *Aurantiochytrium mangrovei* as the only species within the genus containing ameboid form [10], [20] (**Figure 1.1a**). In a liquid media, *Aurantiochytrium* does not form into large colonies but rather, remains dispersed as single cells [10]. *Aurantiochytrium acetophilum* has been recently isolated from a mangrove swamp in Biscayne Bay (Florida, USA) and was shown be extremely tolerant to acetate toxicity with an ability to produce gametes and to possess genes involved in meiosis [15].

1.1.3.2 Botryochytrium

The genus Botryochytrium was created by Yokoyama et al. (2007) [10] to accommodate a single species *Botryochytrium radiatum* (previously: *Ulkenia radiata*) and its distict carotenoid composition. *Botryochytrium radiatum* forms relatively large colonies and possess a well-developed system of EN that connect the cells to form large clusters. Furthermore, *Botryochytrium radiatum* possess an ameboid stage before the zoospores are produced which form a large cluster of botryose shape due to synchronous multipolar budding [10] (**Figure 1.1b**). Recently, *Botryochytrium radiatum* has been shown to contribute to the release of methyl halide from the ocean thus, potentially contributimg to ozone depletion in the lower stratosphere [21].

1.1.3.3 Japanochytrium

First described by Kobayashi and Okubo (1953) [22] the genus *Japanochytrium* contains only a single species (*Japanochytrium marium)* that can be distinguished from the Thraustochytrium genus only by the presence of a swelling (the supsoral apophysis)

just below the sporulating structure and through the release of spores by a single pore in the sporangial wall (**Figure 1.1c**).

1.1.3.4 Parietichytrium

Parietichytrium sarkarianum was first discovered and described as *Ulkenia sarkariana* by Gaertner, A. (1977) [23], and was moved to its own genera *Parietichytrium* by Yokoyama et al., in 2007[10]. It can be distinguished from *Ulkenia* by star-shaped ameboid cells and possession of only one type of pigment, β-carotene, occasionally with a trace of canthaxanthin. As described by Yokoyama et al.,(2007) [10]., it forms large colonies, has relatively well developed EN, and is a unique source of docosatetraenoic acid.

1.1.3.5 Schizochytrium

Due to recent taxonomic rearrangements by Yokoyama and Honda., (2007) [10] *Schizochytrium* contains only one species *Schizochytrium aggregatum*. The genus was first established by Goldstein and Belsky in 1964 [24] to describe thraustochytrids that showed a peculiar mode of reproduction. The cells of *Schizochytrium aggregatum* proliferate by successive binary divisions before each cell transforms into zoosporangia. This mode of division is the main feature that distinguishes *Schizochytrium* genus from other thraustochytrids. In brief, a mature thali proliferates zoosporangia by successive bi-partitioning of the thallus forming diads, tetrads, and clusters before each cell-sporangium releases the zoospores [24] (**Figure 1.1a**). *Schizochytrium* is known for its rapid growth and extensive accumulation of metabolites such as PUFAs(Polyunsaturated fatty acids) and carotenoids, raising considerable interest from the biotechnological industry. Most notably, it is known for its ability to accumulate a high concentration of DHA that can reach more than 30% of total fatty acids in an optimized culture [25]. In terms of morphology, *Schizochytrium* possesses well-developed EN and their colonies are large and yellow-pigmented due to high production of β-carotene [10].

1.1.3.6 Sicyoidochytrium

The genus *Sicyoidochytrium* contains only one species *Sicyoidochytrium minutum* which was removed from the genus *Ulkenia* by Yokoyama et al., in 2007 [10]. The name of the genus derives from the shape of the cell at the final stage of zoospore

formation that resembles grounds of a perennial vine, *Cissus sicyoides*. *Sicyoidochytrium minutum's* EN is poorly developed and during the ameboid stage, the cell wall dissolves completely leaving the protoplast naked and dividing before formation of zoospores [10] (**Figure 1.1b**). Interestingly, *Sicyoidochytrium minutum* may host viruses and has been described as a host to a novel dsDNA viral infection [26].

1.1.3.7 Thraustochytrium

The genus *Thraustochytrium* is the most species rich within the family, with twenty described species [7]. The genus is characterized by production of well-established EN and its particular mode of division. During proliferation, the entire thallus converts into a zoosporangium and the zoospores are released either through a tear in the cell wall or its complete disintegration [27] (**Figure 1.1c**). Notably, two species *Thraustochytrium striatum* and *Thraustochytrium gaertnerium* contain an ameboid stage similar to that by *Ulkenia* genus during which they show bacterivory behavior [14], [22].

1.1.3.8 Ulkenia

The most characteristic feature of the genus *Ulkenia* is the release of ameboid cells before the formation of sporangia [23]. After recent taxonomic amendment by Yokoyama et al., (2007) [10], the genus contains only three species which are *Ulkenia amoboidea*, *Ulkenia visurgensis* and *Ulkenia profunda* with *Ulkenia radiata*, *Ulkenia sarkariana*, and *Ulkenia minuta* recently added to their own genus as *Botryochytrium*, *Parietichytrium*, and *Sicyoidochytrium*, respectively. *Ulkenia* produces small colonies that are orange or pink pigmented due to the presence of astaxanthin, phenicoxanthin, echinenon, and β-carotene. The EN are not well developed and the cell wall disappears completely after the release of ameboid structure (**Figure 1.1b**).

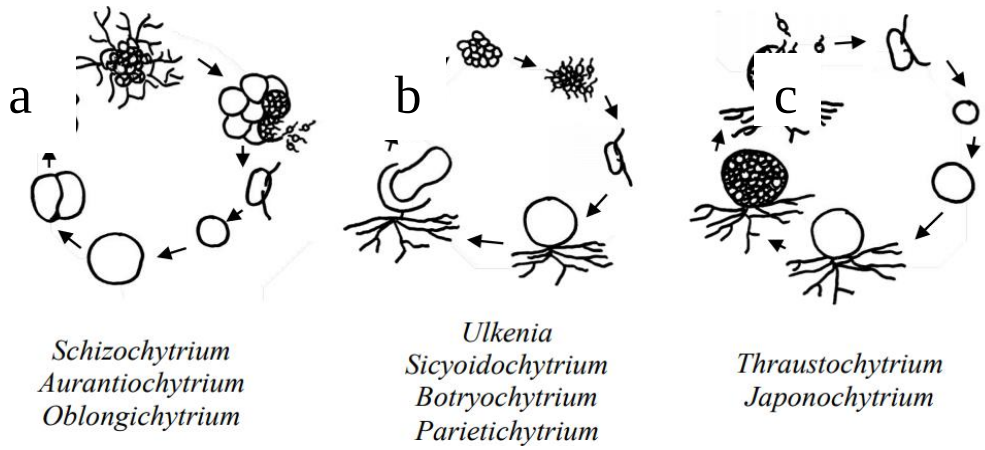

Figure 1.1 Cell cycles representative of genera.
Each cell cycle contains a number of defined stages of the cell growth and zoospore development. **a.** In *Schizochytrium*, *Aurantochytrium* and *Obongichytrium* genera, the mature thali undergoes a successive bi-partition after which each cell forms a sporangium and releases bi-flagellate zoospores. **b.** In *Ulkenia*, *Sicyoidochytrium*, *Botryochytrium* and *Parietichytrium*, a free living ameboidal form is released from a cell wall rupture.Thereafter, the ameboidal body divides internally into a zoosporangium and the zoospores are released into the envirioment.**c.** In *Thraustochytrium* and *Japonochytrium* genus, the cell initially grows in size and then divides its cytoplasm into zoosporangia which upon maturation, releases bi-fligallete zoospores [7].

1.2 Ecology and isolation

1.2.1 Habitat and role in the ecosystem

Thraustochytrids are ubiquitous in estuarine and marine habitats throughout the world. Most notably, they can be found on a variety of organic materials such as decomposing plant and algae material [28], [29], in sediments [30], as a part of plankton [31], on invertebrate tissue [32] or on lesions of infected vertebrates [33]. Due to their mainly saprotrophic nature, thraustochytrids are mostly associated with particulate organic detritus where they use their EN to inhabit surfaces and to extract nutrients from the decomposing matter [34].

The most well-studied habitat of thrasutochytrids is the seagrass and mangrove forest in tropical and sub-tropical areas where they can reach biomass comparable to those of bacteria and other protists [35]. They are believed to be important decomposers of organic matter and possibly a vital food source for other detritus-feeders in the mangrove ecosystem [35], [36], [37]. Indeed, thraustochytrids and Omomycetes are the first colonizers of fallen dead mangrove leaves, and through their production of exogenous enzymes such as xylanase and cellulase, they play an important role in the nutrient cycling [38]. Their role as mangrove decomposers is further supported by the study of Raghukumar et al. (1995) [39] where it was reported that the rate of decomposition of the mangrove leaves correlates with the extent of thraustochytrid colonization. However, in contrast to labyrinthulids, thraustochytrids are not usually found on the vascular plants and living algae, which has been associated with the presence of antimicrobial compounds that limit their growth and propagation on these types of surface[40].

Aside from their role as primary decomposers, some members of the family *Thraustochytriacae* exhibit parasitic growth on marine invertebrates such as octopus or flatworms and may be associated with a devastating disease of the hard clam *Mercenaria* [33], [41]. The disease is caused by Quahog Parasite Unknown (QPX) which appears to be a specific relative of *Thraustochytrium pachydermum* [42]. Furthermore, thraustochytrids have also been isolated from the fecal pellets of the tunicate *Pegea confoederata* in the deep sea [43] and have even been found on the

surface of coral [44]. Thraustochytrids may host viruses such as a double-stranded DNA virus isolated from *Sicyoidochytrium sp*. or a single-stranded RNA virus from *Aurantiochytrium sp*., both of which destructively lyse the host cells [45], [46]. Most notably, since some of the viruses that infect thraustochytrids are also present in some invertebrates and vertebrates, they may contribute to the spread of viral diseases to other organisms.

1.2.2 Isolation and cultivation

Mangroves are a highly variable environment where temperature, salinity, and organic matter concentration tend to fluctuate daily, monthly, or seasonally. Thraustochytrids are highly adaptable to changing conditions and were isolated from many extreme environments such as Antarctica [47] [48], Great Salt lake [49] or the deep sea [43]. The classical method of isolation involves the use of "bait" that takes advantage of thraustochytrid's ability to penetrate and extract nutrients from organic particulates. The most common bait is pine pollen [27], [48], [50], [51], [52]. Successful isolation can be also achieved with other baits such as pollen from the Sweetgum tree (*Liquidambar* sp.) or brine shrimp larvae [52]. In this method, a heat-sterilized bait is usually added either directly to the collected samples or after a serial dilution and left to incubate for up to a week at 20-25℃. Thereafter, the pollen is observed under the microscope for the aggregation of thraustochytrid cells on the bait and it is then transferred onto high nutrient agar (**Figure 1.2**). For organic samples such as mangrove leaves, direct plating of the tissue after saline water wash is preferred [30]. In this method, a section of a leaf or other tissue is carefully rinsed with saline water to remove the bulk of particulates and contaminants and placed directly on the highly nutritious solid media for incubation at 25 ℃ [50], [53]. For seawater samples, serial dilution with a mix of antibiotics can be implemented to isolate thraustochytrids from the water column [18], [54]. This involves, serially diluting the original water sample with sterile seawater and with or without the addition of antibiotics prior to plating on solid media or adding to a liquid media containing antibiotics, and incubation at 20- 28 ℃ [18], [54]. Once colonies of thraustochytrids appear on the agar plates they are then re-plated until a pure culture of thraustochytrids is obtained.

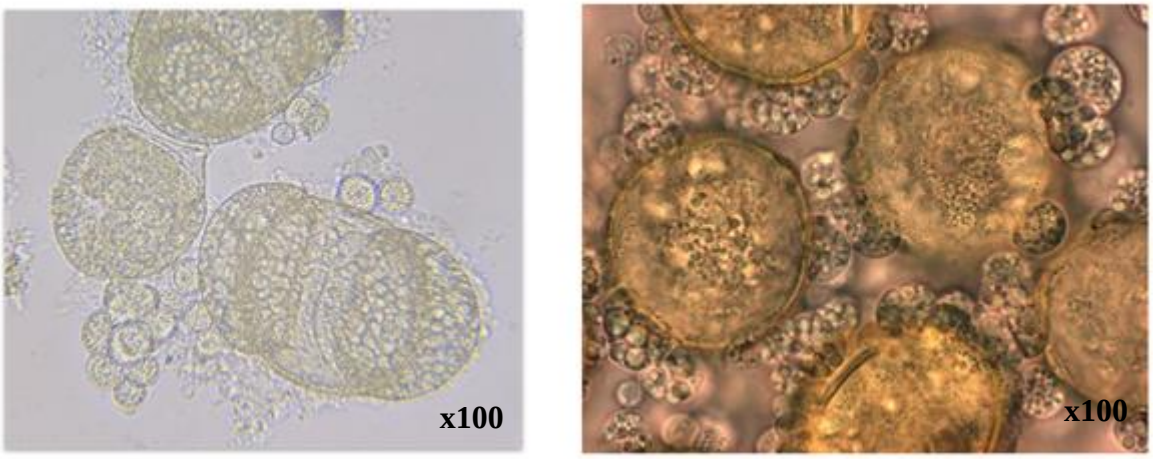

Figure 1.2 Unidentified Thraustochytrids baited on two types of tree pollen. Photograph on the left shows thraustochytrid cells attached to the surface of the pine pollen *Pinus contorta*. On the right, thraustochytrid cells can be seen attached to pollen of black walnut tree *Juglans nigra*, the small dark spherical objects are lipid droplets. (Photograph on the right originates from https://www.masts.ac.uk/media/36000/vf21-masts-report.pdf).

In order to facilitate the isolation of thraustochytrids from environmental samples that are commonly high in fast-growing bacteria and fungi, a mix of antibiotics and antifungal agents is usually added to the baited seawater or media. Bacterial numbers can be controlled with the addition of antibiotics (e.g. penicillin/ streptomycin) which can be combined with broad-spectrum antibiotics such as ampicillin and rifampicin (**Table 1.2**). Since antibiotics target specifically the structure and metabolic pathways present only in prokaryotic microorganisms, they do not seem to influence the growth of thraustrochytrids even at relatively high concentrations [55]. However, due to structural similarities between eukaryotes and especially between fungi and thraustrochytrids, the selection of an effective antifungal agent is much more challenging. Raghukumar (1985) [29], assessed different concentrations of the three most common antifungal agents (cycloheximide, nystatin, and malachite green) and their effect on the isolation of thraustochytrids. Of all antifungal agents used, only nystatin was able to support the growth of thraustochytrids. This can be explained by the mechanism by which these antimicrobials fulfil their function. Cycloheximide inhibits translational elongation in eukaryotes and thus, interferes with protein synthesis whereas malachite green disrupts the function of respiratory chain proteins which results in irreversible inhibition of oxidative phosphorylation [56]. In contrast to cycloheximide and malachite green, nystatin is an ionophore and works by binding to ergosterol which is a major component of fungi cell membrane which is scarce in the

membrane of thraustochytrids [57]. Therefore, in fungi, nystatin works by creating pores in the membrane leading to K+ leakage and apoptosis of the cell [57].

Aside from the selection of antibiotics, media composition is also very important (**Table 1.2**). The media often consists of glucose as a carbon source and yeast extract (GPY media) and/or bacteriological peptone as nitrogen sources (YEPG media) [30], [51], [53]. For isolation of thraustochytrids from invertebrates tissues, a serum containing vitamins, growth factors, sugars, and buffering agents may be supplemented. Many studies used Modified Vishniac media containing 1% fetal bovine serum [27], [59], [60] or SSA media containing 1% of horse serum [52], [62]. Carbon sources from manufacturing by-products like corn steep liquor have also been shown to be successfully incorporated into an isolation media [52] and have an additional growth-stimulatory function due to the presence of other nutrients like amino acids, vitamins, and minerals. Furthermore, Taoka et al., (2008) [62] achieved an increased yield of thraustochytrid isolates by the addition of Tween 80, potassium dihydrogen phosphate, and tomato juice to the standard GYP media. As a surfactant, Tween 80 emulsifies and reduces the size of bigger particles making them more bioavailable to the growing thraustochytrids [62]. Tomato juice is known to contain many growth factors such as pantothenic acid or glutamic acid. Pantothenic acid is a precursor of CoA and supports the growth of eukaryotes such as protozoa and yeast whereas glutamic acid is a very important amino acid that plays many roles in the metabolic processes and during stress response in bacteria [63], [64].

Apart from nutrient composition, another important aspect especially in solid media preparation is the concentration of the solidifying agent such as bacteriological agar. As shown by Rosa et al., (2011) [52], increased concentration of more than 3% of agar has a negative effect on the growth of the colonies. This phenomenon has been linked to the culture media water activity (Aw) suggesting that the increased concentration of agar particles could influence the availability of water of the solid media which consequently, inhibits the growth of proliferating cells. Another factor influencing Aw is the salinity of media which tolerance tends to vary significantly between species and strains. For instance, thraustochytrids isolated from river estuaries where salinity varies significantly due to tidal waves tend to have a much wider salinity tolerance than those isolated from more buffered environments such as deep-sea [44]. However, it has been

shown that despite remarkable ability of thraustochytrids to adapt to halophilic conditions, they are almost always unable to proliferate in the absence of any inorganic salt [65].

Thraustochytrids can be maintained for a longer period either by regular subculturing on adequate media or by cryopreservation. The most commonly used preservative is glycerol. Glycerol is a dehydrating agent with antimicrobial and antiprotease activity making it suitable for long-term storage of microorganisms[57]. To increase the survival of the cells, it can be used in combination with trehalose which as opposed to glycerol does not penetrate cell wall and membrane. Moreover, it has been used widely in mammalian tissue preservation due to reduction of the DNA damage caused by water crystals [30], [66]. Cox et al., (2009) [67] have studied various ways for cryopreservation of thraustochytrids and found that 10% dimethyl sulfide (Me_2SO) and 30% horse serum with 10% cell suspension and 50% culture media is the best choice for the preservation of these organisms. In terms of temperature, Unagul et al., (2017) [30] reported that the survival rates for long-term (more than 12 months) were significantly greater after freezing in liquid nitrogen (−187°C) than storage at −80°C. The overall survival rates of thraustochytrids can also vary between species, with larger vegetative cells and zoosporangia such as those of genera *Parietichytrium* and *Schizochytrium*, appearing to be more tolerant to cryopreservation than smaller cells of species within the genus *Aurantiochytrium*[30].

Table 1.2 Effective isolation strategies employed by other studies for isolation of thraustochytrids from different areas of the world

Study	Location of samples	Samples	Isolation medium	Isolation method	Antibiotics	Antifungals	Isolates
[51]	Iceland	Seaweed, stones, sand	GYP	PP	Penicillin (0.3 g/L) Streptomycin (0.5 g/L)	-	39
[30]	Thailand	Mangrove leaves, seagrasses and seawater	GYP	PP	Penicillin (0.5g/L) Streptomycin (0.5g/L)	-	300
[53]	India	Mangrove leaves and sediment	YEPG	-	Streptomycin 0.1g/L Ampicillin 0.1g/L Kanamycin 0.1g/L	-	11
[60]	China	Mangrove leaves sediment and seawater	MC, MV	PP	Ampicillin 0.5g/L Streptomycin 0.75g/L	Nystatin 0.05g/L	71
[52]	Argentina	Seawater and organic material	MC, SSA, MC-BHB	PP SGP	Penicillin 0.5g/L Streptomycin 0.5g/L	Benomyl 0.05g/l	40
[27]	India	Seawater	MV	PP	-	-	31
[50]	Australia	Seawater samples and sediments containing degraded leaf	YP	PP DP	Penicillin 0.5g/L Streptomycin 0.5g/L Rifampicin 0.05g/L	Nystatin 0.01g/L	13
[68]	Hong Kong	Decaying Kandelia candel leaves	YP	DP	Penicillin 1g/L Streptomycin 1g/L	-	6
[48]	Antarctica	Seawater and sediments	GY	PP	Penicillin 0.3g/L Streptomycin 0.3g/L	-	13
[54]	Tasmania and tropical Queensland	Moist sediments, Seaweeds, dead mangrove leafs	Custom	SD	Penicillin 0.06g/L Streptomycin 0.250g/L	-	36

Isolation medium: GYP: Glucose-Yeast-Peptone media; **YEPG:** Yeast Extract Peptone Glucose media; **MC**: Mar Chiquita media; **MV**: Modified Vishniac; **SSA**: Sterile Seawater Agar. **Isolation method**: **PP**: Pine Pollen, **DP**: Direct Plating, **SD**: Serial Dilution, **SGP**: Sweet-Gum Pollen
PP: Pine Pollen bait; **SGP**: Sweet Gum Pollen; **DP**: Direct Plating method; **SD**: Serial Dilution

1.3 Metabolism and biotechnological application

1.3.1 Lipids

In oleaginous yeast, bacteria and phototrophic microalgae, the storage lipids are dominated by saturated fatty acids, mainly palmitic acid, myristic acid, and stearic acid [59], [69], [70]. The only known oleaginous microorganisms that include long-chain (≥C20) polyunsaturated fatty acids (LC-PUFA) as their storage lipids are thraustochytrids, labyrinthulids, and the heterotrophic dinoflagellate *Crypthecodinium cohnii* [71]. In thraustochytrids, FAs are dominated by DHA (22:6n-2) and palmitic acid (16:0) which may represent up to 65% of total FAs in a cell [72]. The proportion of DHA and the total amount of lipids in thraustochytrids tends to vary between species and genera, *Schizochytrium* and *Aurantiochytrium* genera can accumulate lipids up to 30–50% of their dry weight, with a significant DHA proportion [17]. However, lipid synthesis in these organisms is highly complex because it involves two unique and independently operating pathways. Briefly, in the excess of glucose or other substrates of glycolysis, large amounts of acetyl-CoA are produced which are subsequently converted into malonyl-CoA and tagged for storage (**Figure 1.3**). The first pathway depends on Fatty Acid Synthase (FAS), a multi-domain enzyme complex that catalyzes a strictly ordered number of reactions that convert malonyl-CoA to saturated C16 fatty acids (16:0) by adding two carbons at a time **(Figure 1.3)**. From there, multiple desaturases and elongases synthesize LC-PUFAs such as docosapentaenoic acid (DPA, 22:5n-6), EPA (20:5n-3), or DHA. The second pathway or "PKS pathway" requires a different multi-domain enzyme complex called PUFA synthase which sequentially adds two carbon units to Malonyl-CoA, thus directly converting it into VLCPUFAs (**Figure 1.3**). One of the major differences between these two pathways is the requirement for oxygen presence. Unlike the PUFA pathway, The FAS pathway requires oxygen to desaturate and introduce double bonds in the fatty acid chains [73]. Therefore, the former often proceeds via an"anaerobic" pathway as opposed to the latter "aerobic" pathway. Studies suggest that thraustochytrids use predominantly the FAS pathway to produce saturated fatty acids such as myristic acid and palmitic acid. However, due to the presence of low levels of arachidonic acid and eicosapentaenoic acids, they also appear to express desaturases and elongases [10]. The PKS pathway is much less common and has been reported in some bacteria and *Schizochytrium* [73]. However, it

is speculated that the high DHA producers favor a more direct PKS pathway for the synthesis of DHA in the cell [73].

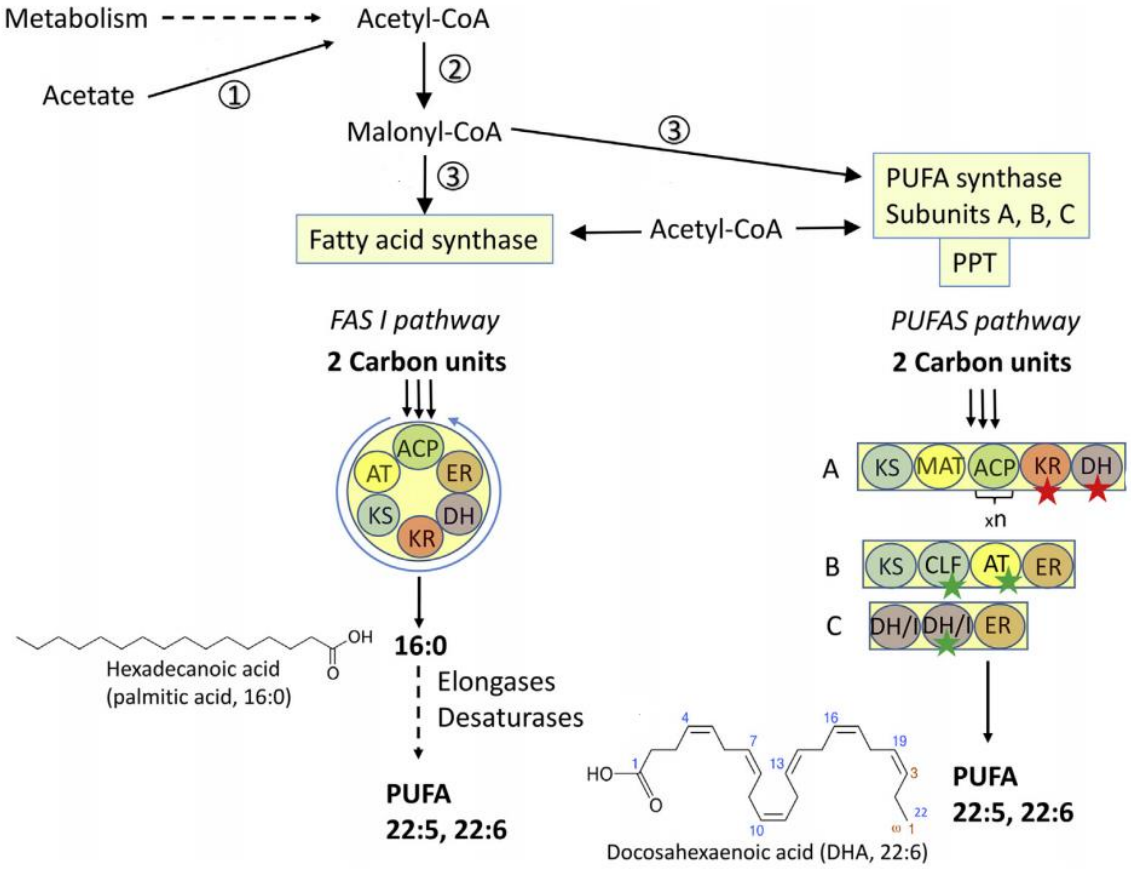

Figure 1.3 PUFA synthesis in thraustochytrids.
The figure shows two independent pathways involved in synthesis of PUFA from the products of aerobic and anaerobic metabolism. Firstly, acetyl-CoA is converted to malonyl-CoA which is the direct substrate for FAS and PUFA pathway. In FAS pathway, a multi-domain enzyme converts Malonyl-CoA into palmitic acid by addition of two carbons at each sequence. Thereafter, palmitic acid becomes elongated and desaturated to PUFAs. A second pathway also called “PUFAs pathway” uses a unique set of multi-doman enzymes that lead to direct synthesis of PUFAS in the absence of elongases and desaturases by sequential addition of 2 carbon units. 1) Acetyl-CoA synthetase; 2) Acetyl-CoA carboxylase; 3) Malonyl-CoA:ACP transacylase. The broken lines indicate that more than one reaction is required to get the final product[73].

The FA production and composition can be increased in thraustochytrids by adjusting the media and the fermentation conditions. The most common method to increase the accumulation of lipids in any oleaginous microorganism is to limit an essential nutrient

- commonly nitrogen and to provide a carbon source in excess [59]. The molecular mechanism behind nitrogen starvation is largely unknown. However, a transcriptome study of *Aurantiochytrium* by Heggeset et al., (2019) [75] revealed that under N-deficient conditions, a significant up-regulation of the FAS pathway occurs which results in increased production of total lipids and DHA fraction in *Aurantiochytrium* genera. To achieve nitrogen starvation, thraustochytrids are grown in nitrogen-rich media until they reach stationary phase, after which a constant supply of carbon source (usually glycerol or glucose) is added daily for a period of time [37], [58], [76], [77]. This strategy has been shown to increase lipid concentration to 40% DW and DHA yield to 1.16 g/L [22], [78]. Nevertheless, despite a significant increase in lipid accumulation, nitrogen limitation results in decreased cell division and biomass [79]. In contrast, phosphate limitation has been shown to produce negligible effects on the cell growth which has been associated with the presence of intracellular supply that provides enough of the element to support the growth [80]. The phosphate concentration can be manipulated by restricting the addition of phosphate sources to the growth media. Ren et al., (2012) [81] reported that in *Schizochytrium*,a 1.4-fold times increase in lipid productivity was due to phosphate limitation than in phosphate-replete media. A similar result was obtained by Jakobsen et al., (2008) [76], where phosphorus and nitrogen starvation of *Aurantiochytrium* culture produced 10-15% more DHA than if only nitrogen was depleted. Another strategy to increase TFA and DHA is using oxygen limitation. Oxygen deprivation suppresses activity of the FAS pathway which depends on oxygen presence. In absence of FAS pathway, the PKS pathway is favored to synthesize higher concentrations of DHA and other PUFAs without being limited by the presence and activity of elongases and desaturases (**Figure 1.3**) [58], [76]. Results from experiments where the temperature was decreased to 10-15°C during the late stationary phase have resulted in increasing total lipids yields with decreasing temperatures. Chodchoey and Verduyn (2012) [82], showed an increase in total fatty acids from 29% to 42% in *Aurantiochytrium mangrovei* in the late stationary phase when the temperature was decreased from 30 to 12°C. However, despite an increase in total fatty acids, DHA has remained stable. A similar result has been obtained by Taoka et al., (2011) [83] in *Aurantiochytrium limacinum* where the total fatty acids increased significantly after the cold shock experiment at 10°C for 72 h, while DHA levels remained stable. Therefore, it appears that the cold-shock treatment is successful in

increasing the total lipids levels but it does not seem to affect the composition of the fatty acids as much as nitrogen or phosphorus depletion and oxygen depravation.

So far, oil is the only product from thraustochytrids that has reached commercialisation. A number of studies have demonstrated their potential as oil producers and particularly as a primary source of DHA. Thraustochytrid oil has been successfully used as a replacement for fish oil in aquaculture feed for many commercially important marine organisms such as shrimps, rainbow trouts, or Atlantic salmon [84], [85], [86]. In aquaculture feed, fish oil provides a source of both dietary energy and PUFA [87]. Usually, a previously extracted oil from thraustochytrids is added directly to the feed to replace other oils such as canola or fish oil but it can also be successfully added at low concentration as whole-cell biomass without any adverse effects in an Atlantic salmon [88]. Thraustochytrid oil is particularly valued due to its high LC-PUFA content as opposed to other cheap, plant- or animal-derived oils which fail to satisfy the requirement for LC-PUFA in the fish diet [87]. Furthermore, in contrast to other LC-PUFA producers such as green microalgae, thraustochytrids effectively bypass the technical difficulties and high costs associated with the cultivation of autotrophic organisms in photobioreactors. Aside from aquaculture feed, thraustochytrid LC-PUFA rich oils were also reported as a potential source of DHA for a human diet [37].This has led to significant research interest in part due to health benefits associated with the inclusion of LC-PUFA in the human diet [90], [91]. Notably, of all LC-PUFA, DHA is essential for the normal development of neural tissues in infants and the maintenance of brain functions in adults [92], [93]. Furthermore, the omega-3 fatty acids EPA and DHA supplementation during pregnancy has led to decreased immune responses in infants and lower incidence of allergies [94].

Globally, fish production reached 179 million tonnes in 2018 with 46% and 52% of total production used for aquaculture feed and human consumption, respectively [95]. Since the fish population has been steadily declining due to overfishing, they will not be able to provide enough oil to sustain the constantly growing human population [96]. Therefore, thraustochytrids offer a promising alternative with an additional added value of relatively higher DHA and much cheaper production costs than other microbial sources.

1.3.2 Extracellular enzymes

Heterotrophic nutrition of thraustochytrids occurs through their well-developed ectoplasmic net system which possesses many hydrolytic enzymes in the outermost layer of plasmalemma and can be secreted to the environment to digest organic matter [73]. Due to their role as decomposers in the coastal ecosystem, they are capable of producing many enzymes involved in the breakdown of proteins, carbohydrates, and lipids such as cellulase, protease, lipase, amylase, gelatinase, urease, and phosphatase [97], [98], [99]. The presence of proteases is common across all species and most of thraustochytrids tested showed high activity of lipases, phosphatases, and ureases [27], [98], [99], [100]. However, the synthesis of chitinases and cellulases tends to vary between species. Indeed, chitinase which is a structural part of the exoskeleton of arthropod zooplankton has been so far found in *Thraustochytrium stratium* and two unidentified species of *Parietichytrium* and *Botryochytrium* genera [27], [98], [100]. The cellulase activity has been reported in almost all thraustochytrid genera including *Aplanochytrium, Botryochytrium, Oblongichytrium, Parietichytrium, Sicyoidochytrium, Thraustochytrium*, and *Ulkenia* with *Aurantiochytrium mangrovei* found to produce cellulase in a co-culture with detrital mangrove leaves [65]. However, other studies provided contrasting results, Taoka et al., (2009) [98] detected no cellulase activity in six species from *Thraustochytrium, Schizochytrium*, and *Aurantiochytrium* genera. Similarly, transcriptome data from Song et al., (2018) [100] and Lin et al., (2020) [101] from different species of *Aurantiochytrium, Schizochytrium, Parietichytrium,* and *Botryochytrium* showed no specific polysaccharides-hydrolyzing enzymes that would enable them to utilize cellulose and amylose. Nevertheless, more data is required to fully elucidate the differences in enzymatic profiles between thraustochytrids and their potential association with their roles as nutrient scavengers in the coastal ecosystem.

In terms of industrial application, microorganisms are favored as sources for industrial enzymes due to their fast growth rates, availability, and ability to use recombinant DNA technology to manipulate enzyme yields [102]. Some of the most industrially important enzymes include amylases, cellulases, chitinases, and ligninases. Amylases are enzymes that catalyze the hydrolysis of glycosidic bonds in starch converting them into simple sugars such as glucose or maltose [103]. Amylases are of great importance in the brewing and food manufacturing industry making up approximately 25% of the world enzyme market [104]. They have been obtained from a variety of plants and

microorganisms such yeast, bacteria, several fungi, and actinomycetes but bacterial and fungal sources are undeniably dominating the industrial application [104]. Another industrially important enzyme, cellulase encompasses several endos- and exo-glucanases that release monosaccharides by breaking β-1,4-linkages in the cellulose polymer [105]. According to Market Watch (CAS 9012-54-8), the global market for cellulase is expected to rise at a significant rate with its major application in the textile, animal feed, paper, and pulp as well as bioethanol industry. Cellulases are extracted from a large diversity of fungi and bacteria during their growth on cellulosic materials and include many genera such as *Cellulomonas*, *Trichoderma*, *Clostridium* or *Aspergillus* [106]. Chitin is the second most common polycarbohydrate in nature after cellulose and a major building block of exoskeletons of insects, crustacean shells, and the cell wall of fungi [107]. Microbial chitinases have been produced by various industries at a mass-scale due to their antifungal and insecticidal properties. Furthermore, attempts were made to isolate and introduce bacteria-derived chitinase genes into agricultural plants to minimize the damage caused by various phytopathogenic fungi [108]. The most common microbial sources of chitinase derive from bacterial species belonging to *Aeromonas*, *Serratia*, and *Bacillus* genera. In terms of thraustochytrids, Brenova et al., (2013) [109] filed a patent for isolation and characterization of the cbh1 gene from *Schizochytrium aggregatum* which has the potential to augment cellulose digestion in *Saccharomyces cerevisiae* however, as of the year 2020, no thraustochytrid-derived enzyme is used for commercial purposes. Nevertheless, considering their fast growth and lack of biotoxins, thraustochytrids offer a great alternative to currently industrialized microbial sources.

1.3.3 Antimicrobial agents and biosurfactants

Thraustochytrids are capable of inhabiting organic matter-rich environments where they must compete for nutrients with other fast-growing microorganisms such as bacteria and fungi. However, the studies assessing antimicrobial activity in Thraustochytrids are scarce. The most recent publication by Ishibashi et al., (2019) [99] analyzed a genome of *Aurantiochytrium limacinum* and identified novel lipases that produce lysophospholipids. These lipophospholipids identified as 1 and 2-acyl lysophospholipids showed antibiotic potential particularly against gram-positive bacteria. Another study by Kalidasan et al., (2015) [110] reported the antibacterial activity of unidentified thraustochytrids against clinical human pathogens from cell

crude extracts in different organic solvents. However, the study failed to determine the active compound responsible for recorded antimicrobial activity. In terms of industrial application, antimicrobial compounds including antifungals and antibiotics were valued at USD 2.2 billion in 2019 (Market Analysis Report, 2020). Their high value is linked with their use as additives in packaging, paints, coating, and food which protect these products against spoilage bacteria and biodegradation.

Similar to antimicrobial compounds, biosurfactants production in thraustochytrids is poorly understood with no published study to date. However, studies in oleaginous fungi of *Rhodotorula* and *Yarrowia* genera showed that lipid accumulating microorganisms can produce biosurfactants at quantities surpassing even bacteria [111], [112], [113], [114], [115]. Moreover, thraustochytrids are well-reported producers of exopolysaccharide (EPS) which were shown to possess biosurfactant properties [116], [117]. Microbial biosurfactants are extracellular compounds that reduce surface tensions in hydrophobic/hydrophilic phases [118]. As per Market Watch forecast, microbial biosurfactants are predicted to reach USD 23 million by 2026, from USD 18 million in 2020 due to their growing importance in bioremediation of oil and heavy metal pollution.

1.3.4 Scope and aims of the project

Despite the growing importance of thraustochytrids and significant attention received in recent years, there is currently very limited data regarding biodiversity and biotechnological potential of Scottish strains. The only study focused specifically on thraustochytrids native to the British coastline was conducted by Marchan et al.(2017) [119] on strains isolated from necrotic tissues of *Eledone cirrhosa* and skin lesions from *Oncorhynchus mykiss*. The lipid analysis of isolated thraustochytrids showed a particularly high DHA proportion of 54-60.7% of total fatty acid. Indeed, the advantage of temperate climate thraustochytrids in high oil accumulation has been proven by similar studies where the relative level of total PUFA or DHA was compared with tropical strains by Lee Chang et al., (2012) [54] and Bowles et al., (1999) [120], respectively. Furthermore, the lack of published data concerning the production of other industrially valuable products such as extracellular enzymes, antimicrobial compounds, or biosurfactants from temperate climate thraustochytrids suggests a gap in knowledge regarding the biotechnological potential of these microorganisms.

The project aimed to isolate thraustochytrids from Scottish coastlines and to screen them for the production of lipids and other high-value compounds. As per **Figure 1.4**, the experimental workflow for this study involved sampling of multiple locations along the Scottish coast, isolation using pollen baiting technique, molecular identification with 18S rDNA sequencing, and conducting screening experiments for presence or quantity of lipids, extracellular enzymes, total proteins, antimicrobial agents and biosurfactants using sulfo-phospho colorimetric assay, substrate digestion plates, Bradford assay, disc diffusion assay, and emulsification assay, respectively. An additional experiment was conducted to identify the stationary phase of isolated thraustochytrids in the media used in the study through measuring a daily fluctuation in optical density of a sample measured at a wavelength of 600 nm (not shown the workflow in **Figure 1.4**)

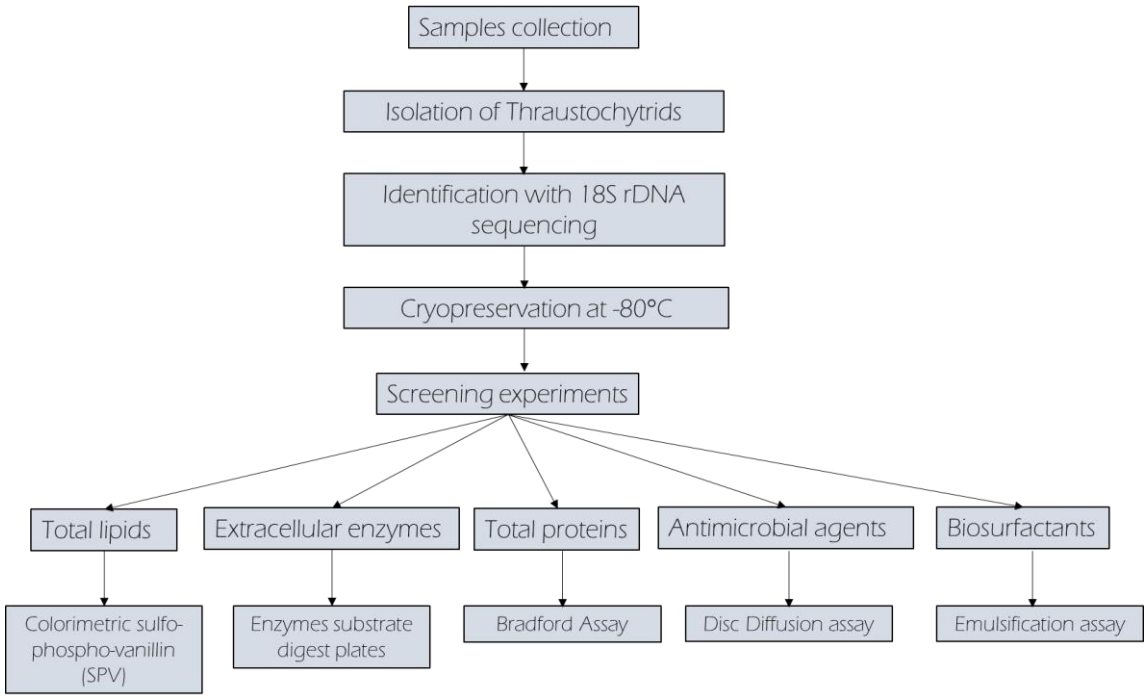

Figure 1.4. Experimental project plan for bioprospecting of Scottish Thraustochytrids. Workflow detailing the sequence of experiments that will be conducted during the project.

Chapter 2- Methods and Materials

2.1 Field sampling

Samples of seawater, sediment, and dead plants were collected from 13 locations along the Scottish coastline between January and May 2019. Coastal saltmarshes were selected for sampling due to the presence of stagnant water pools and creeks that tend to accumulate a high concentrations of organic matter (Haynes, 2016) [140]. The sampled saltmarshes were located near breweries (Wigtown beach), oil refinery (Skinflats, Blackness castle), adjacent to sea a loch (Loch Fyne), fisheries (Filshie J Farm) or at a river estuary (Tyninghame bay, Aberlady bay, Forth Alloa, Piltanton Burn, Kilninver Beach, Skyreburn Bay Beach, and Dunbeg Beach). Five to fifteen samples were collected at each location and the GPS position, pH temperature and salinity were recorded (**Table 2.1**). pH was recorded with ETI 8000 pH Meter (ETI, UK) and temperature and salinity were measured with HI-98319 waterproof salinity and temperature tester (HANNA, USA). Samples were placed in sterile 50mL Falcon tubes containing a standard antibiotic and antifungal mix of 300mg/L of penicillin (Melford, UK), 500mg/L of streptomycin (Melford, UK), and 10mg/L of nystatin (Melford, UK) and seawater from the sampling location. Tissue samples such as leaves, moss, or animal carcass were washed with a sterile saline solution before being placed in the sampling tube to remove sand and other debris. The tubes were transported to the laboratory within 24h from the collection and were stored at 4°C until further use for isolation of thraustochytrids.

Table 2.1. Latitude and longitude of sampling locations that occurred between January to May 2019

Location	Latitude	Longitude
Seafield beach	55.969453	-3.135192
Lindesfarne Nature Reserve	55.671241	-1.799
Inner Tyningham Bay	56.023	-2.59
Aberlady Bay	56.011381	-2.862931
Forth Alloa	56.073727	-3.761432
Skinflats	56.027838	-3.719759
Blackness castle	56.005379	-3.517234
Loch Fyne	56.271223	-4.926928
Piltanton Burn	54.866369	-4.84515
Filshie J Farm	55.971157	-4.688206
Wigtown beach	54.866562	-4.428855
Skyreburn Bay Beach	54.864529	-4.217784
Kilninver Beach	56.34458	-5.51014
Dunbeg Beach	56.45116	-5.42631

2.2 Isolation of Thraustochytrids

Isolation was performed within 48 h from sampling. In this project, a modified pine pollen baiting technique from Rosa et al., (2011)[52] was used to isolate thraustochytrids from the collected samples. In brief, 1mL of seawater, biomass, or sediment was removed from each collected sample and placed in a 25mL universals tube containing 9mL of autoclaved seawater (Peacock salt, UK) to make up a 1:10 dilution. The seawater salinity was the same as measured on the site and antibiotics and

antifungals were added to reach the same concentration as in the sampling tubes (300mg/L of penicillin, 500mg/L of streptomycin, and 10mg/L of nystatin). Fifteen milligrams of heat sterilized pine pollen (24h at 75° C) was added to each tube to encourage the colonisation of thraustochytrids. Thereafter, the universals tubes were incubated at a room temperature (20 °C). Subsamples of pollen were removed daily after three days of incubation to inspect for growth of Thraustochytrids. After a week of incubation, the content of the universals tube was then filtered through a Whatman filter number 4 (Whatman, USA), and to remove contaminating microorganisms, the pollen attached to the filter paper was thoroughly rinsed with a 100mL of 2.5% of seawater (25g of Peacock salt in 1L of distilled water, autoclaved) with the same concentration of antibiotics and antifungal mix as before. Thereafter, the filter paper was removed from the funnel and pressed against a solid MC-BHB media plate until all the pollen was transferred to its surface. The MC-BHB is a nitrogen-high isolation media described by Rosa et al., (2011) that can facilitate the growth of thraustochytrids. The MC-BHB media contained (as % w/v) 0.1% glucose, 0.05% bacteriological peptone, 0.05% yeast extract, 0.05% of monosodium glutamate (MSG), 0.1% of gelatine hydrolysate, 0.05% of corn steep liquor, 1.75% of artificial sea salt, 1.75% of brain-heart broth and 2% agar dissolved in 1L of distilled water. All ingredients were purchased from Melford's (UK) apart from glucose, corn steep liquor, gelatine hydrolysate, and artificial sea salt which were purchased from Kent Foods Ltd. (UK), British Aqua feeds (UK), Organotechnie (France), and Peacock salts (UK), respectively. The MC-BHB media also contained the previously described antibiotic and antifungal cocktail and its pH was adjusted to 7 before autoclaving (121° C, 20 minutes). The plates with pollen were incubated at 25° C for up to two weeks and inspected daily for the appearance of thraustochytrids colonies. Once colonies started to show up on a plate, a single thraustochytrid-like colony was picked with a sterile loop, checked under the microscope for Thraustochytrid features, and streak-plated on MC-BHB media plates with antibiotics. The process was repeated until pure culture of thraustochytrid isolate were obtained.

2.3 Cultures maintenance and preservation

Thraustochytrid cultures were maintained on a modified GPY media containing 2% glucose, 2.5% salt, 0.25% MSG, 0.05% yeast extract, and 0.5% bacteriological peptone containing the antibiotic mix (300mg/L of penicillin, 500mg/L of streptomycin) with a reduced concentration of nystatin to 5mg/L, and the pH adjusted to 7.4 before

autoclaving. The cultures were kept incubated at 25° C and replated every week. For long term preservation, a three-day-old colony was picked up from the plate with a loop and transferred into a sterile 1.5mL cryogenic tube (Nalgene, USA) containing 700µL of a modified GPY media and 300µL of autoclaved glycerol (Melford, UK). The tubes were vortexed briefly stored at -80° C. The isolates were preserved in triplicate.

2.4 Molecular identification

2.4.1 Genomic DNA extraction

Cell lysis, DNA extraction, and purification were performed using a DNeasy PowerSoil Pro extraction kit (Qiagen, Germany) as per the manufacturer's instructions. In brief, the pellets were obtained from 2 to 8 mL of 7 days old liquid culture by centrifugation at 10,000 x g for 5 minutes. The supernatant was discarded and 250mg of cell pellet was transferred onto PowerBead Pro Tube and re-suspended in 800µL of a lysis buffer. To break the cells, the tubes were vortexed at maximum speed for 10 minutes after which they were centrifuged at 13,000 x g for 2 minutes. The supernatant containing a mix of cell debris, proteins, and nucleic acid was then transferred into a clean 2mL microcentrifuge tube and the non-DNA organic and inorganic matter was precipitated with 200µL of Inhibitor Removal Technology™ solution and vortex at maximum speed for 5 s. The tubes were then centrifuged at 13,000 x g for 2 minutes to pelletize non-DNA matter and the supernatant containing nucleic acid was transferred to a clean 2mL microcentrifuge tube (supplied with a kit). For the purification step, 600µL of a high-concentration salt solution was added to facilitate the adhesion of DNA to the silica of the column. Thereafter, the tube was vortexed for 5s and 650µL of the lysate was pipetted directly onto an MB Spin Column. The MB column was then centrifuged at 13,000 x g to retain DNA on the column's silica while the contaminants pass freely through the membrane. The step was repeated twice with the same lysate to remove all the contaminating matter from the MB column membrane. To ensure high purity of the DNA, the MB column was washed with 500µL of EA wash buffer followed by 500 µL with ethanol-based wash solution. The MB column was centrifuged after each wash and collected supernatant discarded. To remove wash solution from the column, the MB column was centrifuged at 13,000 x g for 4 minutes. Finally, the DNA was eluted from the column into a sterile 2mL microcentrifuge tube by adding 100µL of elution buffer (10mM Triss) directly onto a membrane column and centrifuging at 13,000 x g for 2

minutes. The resulted supernatant was validated for the recovery of DNA by gel electrophoresis, as described in section 2.4.4.

2.4.2 Amplification of 18S rDNA gene by the polymerase chain reaction

Amplification of the 18S rDNA gene was performed using polymerase chain reaction (PCR). Primers (**Table 2.2**) were used as per the manufacturer's instructions (MiAlgae Ltd.). Briefly, the 50 µL PCR reaction mix contained the following: 1 µL of genomic DNA from section 2.4.1, 0.4 µM of each adequate primer (Sigma Aldrich, USA), 10 µL of 5x MyTaq Reaction Buffer (Meridian Bioscience, USA), 25µL of Taq DNA polymerase (Meridian Bioscience, USA) and nuclease-free water (Lonza, Switzerland) added to reach a final volume of 50 µL. A non-template control (NTC) was prepared by replacing DNA in a PCR reaction mix with nuclease-free water (Lonza, Switzerland). PCR amplification was performed using a Biometra thermocycler (Biotron, Germany).

The initial denaturation step was performed at 94°C for 5 min. The 30 cycles started with 1 minute of denaturation at 94 °C. The annealing conditions were set at 20 sec at 55°C for 1 minute and followed by a 3 minutes extension at 72 °C. A final 3 minutes of extension at 72 °C was used, followed by and cooling down to a hold temperature of 10 °C. The amplification was checked by gel electrophoresis, as described in section 2.4.4.

Table 2.2 Oligonucleotide primers used to amplify and sequence the 18S rDNA gene of Thraustochytrids for phylogenetic analysis.

Primer	Sequence (5' to 3')	Base Pair Position	Direction	Reference
EukA	AACCTGGTTGATCCTGCCAGT	1–20	Forward	[6]
EukB	TGATCCTTCTGCAGGTTCACCTAC	1780–1800	Reverse	[6]
FA2	GTCTGGTGCCAGCAGCCGCG	555-574	Forward	[141]
FA3	CTTAAAGGAATTGACGGAAG	1125-1144	Forward	[141]
RA1	AGCTTTTTAACTGCAACAAC	605-624	Reverse	[141]
RA2	CCCGTGTTGAGTCAAATTAAG	1171-1191	Reverse	[141]

2.4.3 PCR product purification

To remove nucleotides and primers remained after the PCR procedure, the products were purified with Wizard® SV Gel and PCR Clean-Up kit (Promega, UK) as per manufacturer's instructions. In brief, after the PCR reaction, an equal amount of Membrane Binding Solution was added to the PCR amplification product. Thereafter, the PCR product was transferred onto a supplied Minicolumn assembly and incubate at room temperature for 1 minute to allow DNA biding to the column. The solution was centrifuged at 13,000 × g for 2 minutes and the flowthrough containing PCR reagents residues was discarded. PCR products were then washed by adding 700µL of ethanol-based Membrane Wash solution and minicolumn was centrifuged at 13,000 × g for 2 minutes. The step was repeated with another 500µl membrane wash solution and centrifuged for 10 minutes at the same speed. The residual ethanol from the membrane wash solution was then evaporated off by centrifugation of the column assembly for 1 minute at the same speed with the microcentrifuge lid opened. DNA was then eluted into a sterile 1.5ml microcentrifuge tube (Eppendorf, Germany) by addition of 30µl of Nuclease-Free Water to the Minicolumn and incubation at room temperature for 1 minute. The column was centrifuged at 13,000 × g for 2 minutes and the eluted PCR product was stored at –20°C until use.

2.4.4 Gel electropherisis

Gel electrophoresis was performed as per MiAlgae company's protocol. Briefly, 2% agarose gel with 1.5 µl of SYBR™ Safe DNA Gel Stain (Invitrogen, USA) in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) was used. To allow visual tracking, 1µl of 6X TriTrack DNA Loading Dye (Invitrogen, USA) and 4 µl of deionised water (Lonza, Switzerland) were added to each sample. Furthermore, 1µl of 100bp DNA ladder (Invitrogen, USA) was run alongside DNA and PCR products to define their size. The gel was resolved at 90V and a UV transiluminator was used to visualize the gel products.

2.4.5 Densitometry

To determine the concentration of the PCR products, the "Band Analysis" tool of ImageLab software version 4.1 (Bio-Rad, UK) was used as per ImageLab Software User guide (Chapter 5, Analysing Images) to quantify the background-subtracted PCR

band densities of amplicons. The concentration of the DNA was determined on an unaltered, computer-scanned images by comparing the amplicon's bands with the relevant ladder densities.

2.4.6 DNA sequencing, sequence alignment, and phylogenetic analyses

The 18S rDNA gene amplicons were sequenced using the universal 18S primers EukA and EukB, and four internal primers FA2, FA3, RA1, and RA2 (**Table 2.2**) to ensure full and overlapping coverage of the whole 18S rDNA gene sequence. Sequencing was performed by DNA Sequencing & Services (MRC I PPU, School of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. The sequencing samples contained 80ng of the DNA, 3.2 picomoles of each primer, and an adequate volume of de-ionized water (Lonza, Switzerland) to reach 30µL volume. DNA sequencing chromatograms were analyzed and manually curated using the software Chromas v.2.6.6 (Technelysium Pty Ltd, Australia) and aligned using the online Clustal-Omega alignment tool (EMBL, Hinxton). The Standard Nucleotide BLAST tool on GenBank (NCBI, USA) was used to analyze the consensus gene for each isolate to determine the closest relatives to other thraustochytrid sequences.

2.5 Stationary phase determination

To determine the stationary phase of isolated thraustochytrids, selected isolates were inoculated from a solid medium to 100mL of a modified GYP media and grown in triplicate at 29°C with a shaking speed of 150 rpm for 7 days. The samples of 1mL were taken each day and OD600 was recorded using DR1900 Portable Spectrophotometer (Hach Ltd, USA). The results were plotted with standard deviation values and the curves were generated to connect all data points.

2.6 Lipid quantification

Total lipids were quantified using Sulfo-phospho-vanillin assay that was developed by Charbol and Charonnat in 1937 [121] to allow fast and accurate estimation of total lipids in the patient's serum. The protocol used in this study was adopted from Byreddy

et al., (2016) [122] and allows fast and accurate estimation of total lipids without the need for lipid extraction and cell breaking. Sulfo-phospho-vanillin assay effectivity is based on a chemical reaction between sulfuric acid-treated cell lipids and phosphovanillin solution which leads to a pink pigmentation of the solution. Highly concentrated sulfuric acid breaks the cell wall denaturing the proteins and charring carbohydrates. Furthermore, it reacts with unsaturated fatty acids by forming carbonic ions in place of a double bond [121]. The reaction of carbonic ion and cyclic phosphovanillin compound produce pink color which intensity corresponds to the number of carbonic ions equal to the number of unsaturated fatty acids.

2.6.1 Cell pellet preparation

Selected isolates were inoculated from a solid medium to 40mL of a modified GYP media in sterile 50mL Falcon culture flasks (Corning, USA); each isolate was grown in triplicate at 25°C with shaking at 150 rpm for 7 days. To encourage fatty acid accumulation, glucose was added daily to maintain a media concentration of 20g/L from day 4 until the end of the incubation period. On day 7, the cultures were centrifuged at 10,000 x g for 5 minutes, and the pellet was collected. To remove any residual glucose and media ingredients, the pellet was washed four times with 1x PBS buffer (NaCl: 137 mM

KCl: 2.7 mM, Na2HPO4: 10 mM, KH2PO4: 1.8 mM). Thereafter, the pellet was oven-dried at 40 °C for 24h and the dry cell weight (DCW; g/L) was measured gravimetrically by weighing balance and kept at 4°C for further use.

2.6.2 Colorimetric sulfo-phospho-vanillin assay

To prepare a standard curve, a lipids stock solution was prepared by dissolving 10mg of commercial Canola oil 10mL of 99% chloroform (Sigma Aldrich) to reach a final concentration of 1g/L. Thereafter, different concentrations (15-90 µg) of lipid samples were taken in an acid-washed clean glass tube and filled with chloroform until 1mL (**Table 2.3**). The tubes were incubated at 100 °C for 20 min to evaporate the chloroform and 100 µl of distilled water was added to each tube. Lipid quantification was done by the SPV reaction (described below).

Table 2.3 Table with volumes of stock solution with corresponding lipid content (in mg) and solvent (chloroform) used for lipids standard curve preparation

Lipid weight (in mg)	Volume (uL) of stock solution	Volume (uL) Chloroform
0.015	15	985
0.030	30	970
0.045	45	955
0.060	60	940
0.075	75	925
0.090	90	910

For quantification of lipids in isolated thraustochytrids, 100 µl of deionized water was added to an Eppendorf tube containing 2mg of previously prepared cell pellets from section *2.6.1*. The tubes were then left to soak for 30 minutes after which they were vortexed for 5 minutes at a maximum speed. Once the dry pellet dissolved completely in water, 10µl was transferred into acid-washed pyrex glass tubes (Corning, USA) and filled with deionized water until 100 µL to a final concentration of 20mg/L. The negative control contained 100 µl of deionized water. The SPV reaction was initiated by the addition of 2mL of 98% sulfuric acid (Honeywell, USA) and incubation at 100°C for 10 minutes. After 10 minutes, the tubes were then removed from the heat block and 5mL of phosphovanillin solution was added. Phosphovanillin solution was prepared in advance by dissolving 0.120g of 99% vanillin (Sigma Aldrich, USA) in 20mL distilled water with the final volume adjusted to 100mL with 85% phosphoric acid (APC pure, UK). The tubes were vortexed at maximum speed for 10s and incubated in dark at 37°C for 15-20 minutes. The absorbance was measured at 530nm using DR1900 Portable Spectrophotometer (Hach Ltd, USA).

2.7 Screening for extracellular enzymes

Extracellular enzyme activity was inspected using the modified method of Taoka et al., (2009) [98]. The method employs agar plates and dyes to identify enzyme activities by

zones of clearance on the agar surface, resulting from the degradation of compounds. In brief, the cultures of thraustochytrids were prepared by inoculating a 20mL modified GPY media which was incubated for 4-7 days at 29°C with a shaking speed of 150 rpm. Meanwhile, the plates were prepared by mixing adequate enzyme substrate with 0.025% yeast extract, 0.05% MSG, 2.5% Artificial sea salt, and 1.2% agar. Amylase, cellulase, lecithinase, chitinase, ligninase, and caseinase plates contained 0.2% of dextrose, 0.2% of Carboxymethylcellulose (Fisher Scientific, USA), 10% of egg yolk (EO labs, UK), 0.1% chitin (Sigma Aldrich, USA), 0.4% lignin (Sigma Aldrich, USA) and 0.3% of Casein from bovine milk (Sigma Aldrich, USA), respectively. Thereafter, the prepared media were autoclaved at 120°C for 20 minutes (pH adjusted to 7) with egg yolk emulsion added separately into the autoclaved media to avoid degradation. Each plate was spot-inoculated 3 times with 20 µl of 4-7 days culture of each tested isolate and left to incubate at room temperature for 7 days. Revived cultures procured from DSMZ (Leibniz Institute, Germany) of *Pseudoalteroma aliena*, *Cellulophaga fucicola*, *Arthrobacter agilis*, and *Lentinula edodes* were used as a positive control whereas modified GPY media was used as a negative control. Colloidal cellulose, chitin, lignin, and casein were purchased from Sigma Aldrich (USA), egg yolk emulsion from E&O labs (UK), and glucose from Kent foods (UK). After the incubation period, the plates were flooded with dyes and solutions to reveal zones of enzyme digestion. For amylase detection, plates were flooded with Lugol's iodine solution (SLS, UK), 1% Congo Red solution (SLS, UK) was used to stain plates with cellulose and chitin, 30% trichloroacetic acid (Sigma Aldrich, USA) was used for casein, lignin was stained with 0.5% of potassium ferricyanide and 0.5% of ferric chloride (Sigma Aldrich, USA) and for detection of lecithinase, no dye was necessary as the halos were visible without staining.

2.8 Total protein quantification

Total protein was quantified by the colorimetric Bradford assay following a modified method developed by Tran et al., (2020) [37]. In brief, cultures of thraustochytrids were grown in triplicate (as in section 2.5) and cell pellets obtained by centrifugion at 10,000 x g for 5 minutes. Then, 10mg of wet pellet was added to a 2mL Eppenforf tube containing 1mL of 0.1M NaOH and vortexed for 1min. In order to break the cells, tubes were left on ice for 1h until frozen and then vortexed for 20 minutes at maximum speed. The process was repeated 3 times. The mixture was then centrifuged for 5 minutes at

10,000 x g and the supernatant was filtered through a sterile 0.45 µm syringe filter. Then, 100uL was transferred into an acid washed glass tube and 3mL of Bradford reagent solution (Sigma Aldrich, USA) was added. The tubes were vortexed thoroughly and left to incubate at room temperature for 15 minutes and the absorbance was measured at 595 nm. Protein standard curve was prepared with different concentrations of 100 mg/L of bovine serum albumin (SLS, UK) in 0.15M NaCl and used to quantify the total protein content of the isolated strains.

2.9 Emulsification assay

The cells were inspected for the presence of biosurfactants with a modified protocol of Gutierrez et al., (2008) [123]. In brief, 1mL sample of culture's broth was centrifuged at 13,000 x g for 5 min and 1mL of supernatant was mixed with an equal volume of 99% n-hexadecane (Sigma-Aldrich, USA) in an acid-washed glass tube. The tubes were then manually shaken for 15s and vortexed at maximum speed to homogeneity. The mixture was left to stand for 10 minutes and then the mixing process again repeated. The height of the emulsion layer was measured and compared to the original height of the oil after allowing the mixture to stand for 10 minutes and then after 24h at room temperature.

2.10 Antimicrobial activity screening

The protocol for antimicrobial activity was developed with the guidance of Professor Marcel Jaspars from the University of Aberdeen, Scotland, and adapted from methods reported by Kalidasan et al. (2015) [11] and Hussain et al. (2009) [124]. The protocol used here was modified so that it involves a separate extraction of the cells and broth extracts and subsequent plating on the lawn of bacteria (see below for species used). Thereafter, following an incubation period, the plates are inspected for the presence of halos indicating a zone of bacteria inhibition. In brief, cells were grown to the stationary phase as outlined in section 2.5. Thereafter, the cultures were centrifuged at 10,000 x g for 5 minutes with pellet and broth separated for distinct extraction procedures. The broth was filter sterilized with a 0.2 µm syringe filter, and 3mL was transferred into an acid washed glass tube with a screw-up. An equal amount of 100% ethyl acetate (Fisher Scientific, USA) was added to the broth and the tubes were left shaking at 120rmp for 24h at room temperature. The broth was extracted in ethyl acetate three times. The total ethyl acetate was then collected and stored at -20°C until further use. The pellet from

the same culture was extracted in methanol. In brief, cell pellets were freeze-dried and weighed before transferring into a 5mL volume Eppendorf tubes (Eppendorf, Germany) containing 1mL of acid-washed 0.5mm zirconia beads (Cole-Parmer, USA). Each tube containing the pellet with beads had 3mL of 99.85% methanol added and was vortexed at maximum speed for 20 minutes in 5 min bursts. Afterward, the tubes were incubated at 28.0 ℃ and 131rpm for 24h. The process was repeated three times with methanol replaced with a fresh one after each incubation period. The methanol and ethyl acetate was concentrated in a rotary evaporator. Obtained extracts were then used to immerse 6mm blank antimicrobial susceptibility discs (Thermofisher, USA) by transferring 30 µL of the extract onto a disc and leaving them for 1h to evaporate the solvent. For bacteria, 6mm discs containing 10µg of Gentamicin (Fisher Scientific, USA) was used as a positive control. For yeast, blank 6mm discs were immersed in 99% ethanol solution of 50mg/mL of nystatin for positive control, and methanol and ethyl acetate were used as negative controls (Venables et Russel, 1975). The bacterial and fungal lawns were prepared with cultures procured from the DSMZ (Leibniz Institute, Germany). Microorganism selection was based on using gram rating (in bacteria), safety (Risk group 1 microorganism were selected), and difficulty of culture (aerobic microorganisms were favored). Therefore, for gram-positive bacteria, *Lactobacillus Plantarum*, *Bacillus subtilis*, and *Bacillus licheniformis* were selected. Gram-negative bacteria included *E.coli K12* and *Pseudomonas fluorescens* and only *Saccharomyces cerevisiae* was selected for antifungal screening. *Bacillus subtilis*, *Bacillus licheniformis*, *E.coli K12*, and *Pseudomonas fluorescens* were grown at 30°C in simple nutrient media containing 0.8% of bacteriological peptone whereas *Lactobacillus plantarum* was grown at 30°C in a modified MRS media (2% peptone, 0.5% yeast extract, 2% glucose, 0.1% of Tween 80, 0.2% of K2HPO4, 0.5% of Na-acetate, 0.2% of Ammonium ferric citrate, 0.02% of MgSO4 x 7 H2O and 0.005% of MnSO4 x H2O). *Saccharomyces cerevisiae* was grown at 25°C in a media containing 0.3% yeast extract, 0.3% bacteriological peptone, 0.5% soybean peptone, and 1% glucose. The lawn of bacteria and fungi was created by spread plating 100 µL of 24h liquid culture of each microorganism. Thereafter, the discs containing extracts (as described above) were placed on an adequate plate with sterilized forceps. Plates containing bacteria were incubated at 30°C and those containing fungi were kept at room temperature for 48h-72h. The plates were then investigated for the presence of an inhibition zone around the discs and the diameter of the area was measured.

2.11 Statistical Analysis

Statistical analysis was performed using MiniTab18 (Minitab Inc., State College, USA). Anderson-Darling normality test was performed on a triplicate protein concentration from the section to check for normal distribution. The intergroup differences between protein concentration in cell biomass and both extracts were evaluated with a two sample t-test.

Chapter 3- Results

3.1 Sampling and Isolation

Eighty-three samples were collected in total from all fourteen locations around the Scottish coast. The salinity, temperature as well as pH varied between the sampling locations (**Table 3.1**). The lowest salinity of 6.7ppt was recorded in Forth Alloa and the highest of 32.5 ppt in Aberlady Bay and Skyreburn Bay Beach. In terms of temperature, the lowest 5 °C was recorded at Seafield beach and the warmest 18 °C at Aberlady Bay. The pH was much more stable and remained between 6.45 and 8. The samples varied in their composition, with 35 containing decomposing macroalgae particularly from the order Fucales such as *Fucus vesiculosus* as well as other genera *Laminaria* and *Ulva*. Sediment was also common among the samples with 34 of them containing sediments from various layers of sand and the bottom of the water pools. These were followed by water (21 samples), animal detritus (5 samples), bird's feces (2 samples), decomposing wood (1 sample), and dead mollusk (1 sample).

In total, 29 samples out of 83 contained thraustochytridslike cells and 20 were successfully isolated with pine pollen baiting and cultured into purity (**Figure 3.2**). From twenty-nine samples containing thraustochytrids, eleven consisted of sediment, eight of animal detritus, seven contained decomposing macroalgae, and 3 samples comprised of seawater (**Figure 3.1**). However, in terms of successful isolation and axenic culture, most of the isolated thraustochytrids were obtained from decomposing macroalgae and seawater (100% of thraustochytrids isolated) as well as animal detritus (87.5%) and sediment (36.36%) (**Figure 3.1**). Furthermore, the majority of isolated thraustochytrids derived from three saltmarshes namely, Kilninver saltmarsh, Skyeburn Bay Beach, and Aberlady Bay which all had salinity over 32ppt. No thraustochytrids were found in saltmarshes that were located near oil refinery (Skinflats and Blackness castle) or that had salinity lover than 10ppt (Filshie J Farm, Forth Alloa, Lindesfarne Nature Reserve (Figure 3.2). Interestingly, only one thraustochytrid strain (Tynin04) was isolated from the samples that were collected between January and February with most of the Thraustochytrids isolated from the samples obtained during April and May (**Table 3.1 & Figure 3.2e**)

Table 3.1 Number of samples collected for each sampled location with salinity, pH and temperature and period of collection (in months).

Number of samples collected	Location	Salinity (ppt)	pH	Temperature	Month
7	Seafield beach	30.3	8	5 °C	January
6	Lindesfarne Nature Reserve	3.9	8.01	8 °C	January
11	Tyninghame Beach	23.6	7.75	7.80 °C	January
5	Aberlady Bay	32.5	7.31	18 °C	May
5	Forth Alloa	6.7	6.49	5°C	February
5	Skinflats	17	6.35	7.20 °C	February
5	Blackness castle	26	7.95	6.10 °C	February
5	Loch Fyne	4.7	8	10°C	April
5	Piltanton Burn	20.6	6.45	15°C	April
5	Filshie J Farm	25.3	7	12°C	April
5	Wigtown beach	27.9	7.5	18°C	April
5	Skyreburn Bay Beach	32.5	7.31	19°C	April
8	Kilninver Beach	32.1	7	12.40 °C	May
6	Dunbeg Beach	28.8	7	13.00 °C	May

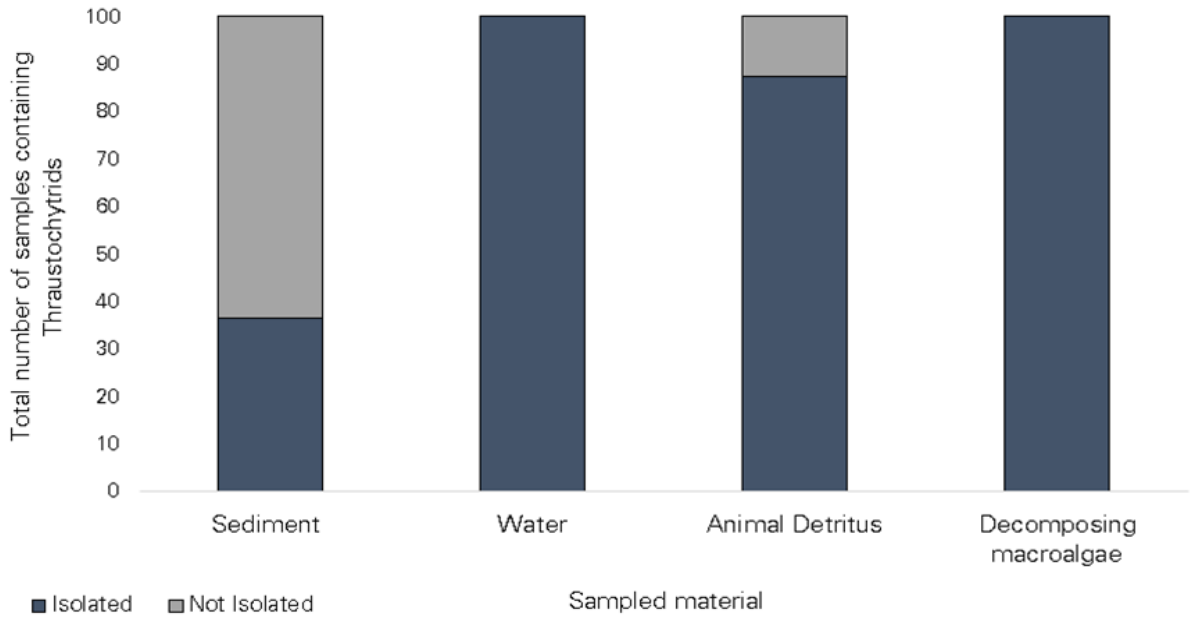

Figure 3.1 Percent of Thraustochytrids isolated from the total number of samples per each collected material.
A stacked bar chart indicating a percent of samples from which Thraustochtrids were successfully isolated per total number of isolates collected from each material.

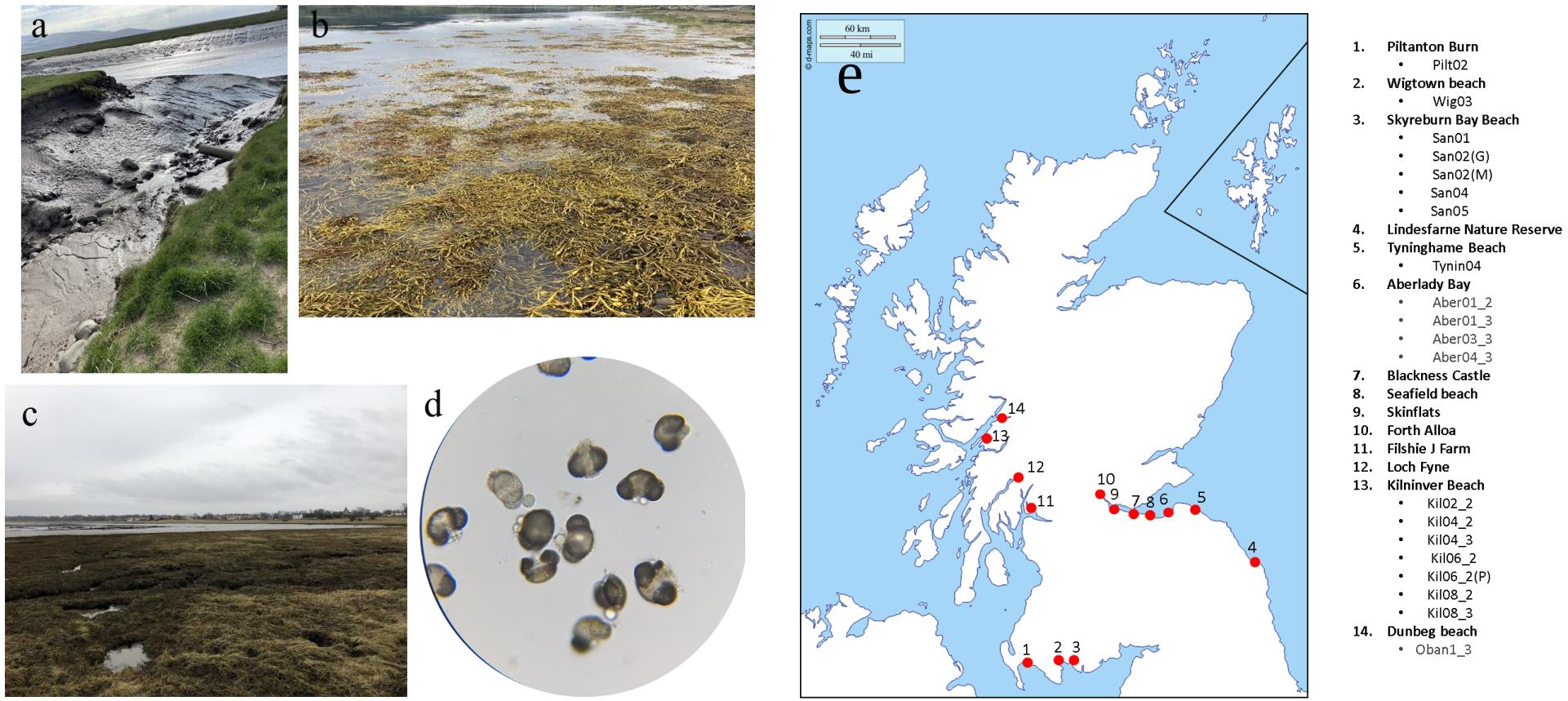

Figure 3.2. Photos of sampled saltmarshes and geographical distribution of twenty isolated Thraustochytrids.
a- Mudflats at Wigtown beach saltmarsh; **b-** Bed of macroalgae at Dunbeg saltmarsh, **c-** Marsh flats at Kilninver saltmarsh; **d-** A microscope photography (x40) of unidentified Thraustochytrid collected from the Aberlady Bay baited on the pine pollen grain; **e-** A map of Scotland pinpointing coastal locations where the samples were collected between January and May 2019.Each successfully isolated Thraustochytrid strain was named after its sampling location and a number indicating the order at which it was collected. (Map adopted from https://d-maps.com/carte.php?num_car=15858&lang=en)

3.2 Molecular identification

The DNA of isolated Thraustochytrids was successfully extracted and all isolates were confirmed to be eukaryotes with EukA and EukB primer set (**Figure 3.3A,B**). Partial and full 18 rDNA sequences were compared with a NCBI (National Center for Biotechnology Information) library using blast alignment which confirmed that all twenty isolates belong to thraustochytrids (**Table 3.2**). All isolates matched with thraustochytrid sequences from NCBI database obtaining high identity scores between 95.56%- 99.85% with E-value equal to 0.00. Majority of strains (n=11) where found to be the members of *Thraustochytrium* genus with *Thraustochytrium kinnei* strains being the most frequently identified (Kil06_2, Kil08_3, Kil04_2, San-02 (M), San04, San05, Kil04_3, Kil08_2, Oban1_3, Tyn_04, Pilt02). Two thraustochytrids were identified with high similarity score over 98% to *Thraustochytrium striatum* (Kil02_2 pink and Kil06_2 (P)). Six isolates were identified as members of *Ulkenia* genus, namely strains of *Ulkenia visurgensis* (San-02 (S), Wig03, Aber01_2, Aber04_3, Aber03_2). San01 is the only thraustochytrid that has matched with *Aurantiochytrium* genus but its species remains undefined.

3.2.1 Isolates selection for screening

Based on sequence similarities produced from alignment data, 8 isolates were chosen for further screening experiments. Oban 1_3, Tynin04, Pilt02 and Kil06_2 closely related to *Thraustochytrium kinnei*, Wig03, San02, Aber01_2 and Aber04_3, which are the strains of *Ulkenia visurgensis*, Kil2_2(P) as *Thraustochytrium striatum* and San01 as *Aurantiochytrium sp*. The aim was to reduce the number of redundant isolates that were either identical or very similar and thus, to avoid screening of the same microorganisms.

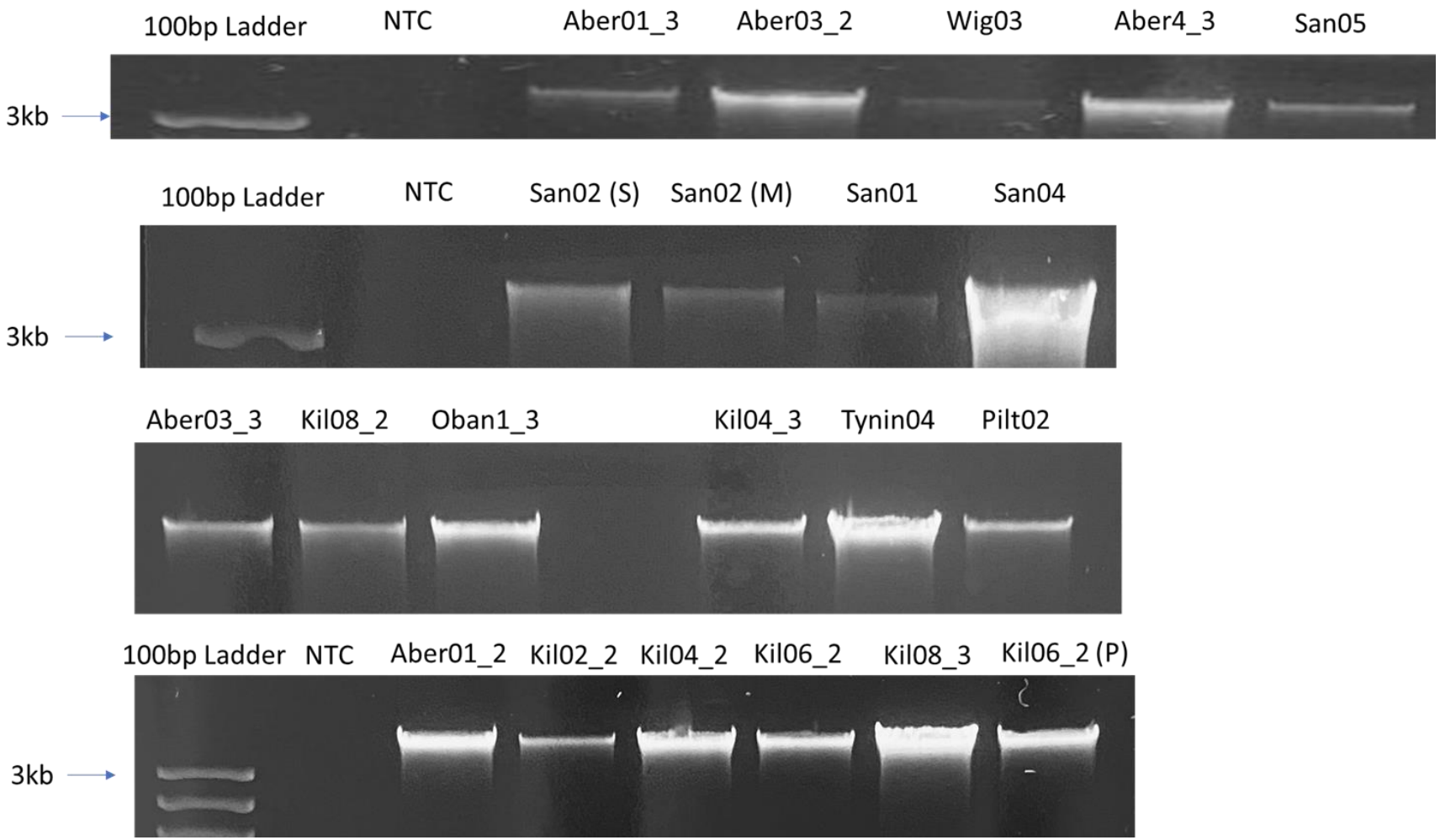

Figure 3.3 A. Two percent agarose gel electrophoresis showing results of DNA extraction from isolated Thraustochytrids. 100bp DNA ladder was used on each gel and NTC lane contains a non-template control. Each band corresponds to the total DNA extracted from the cell biomass.

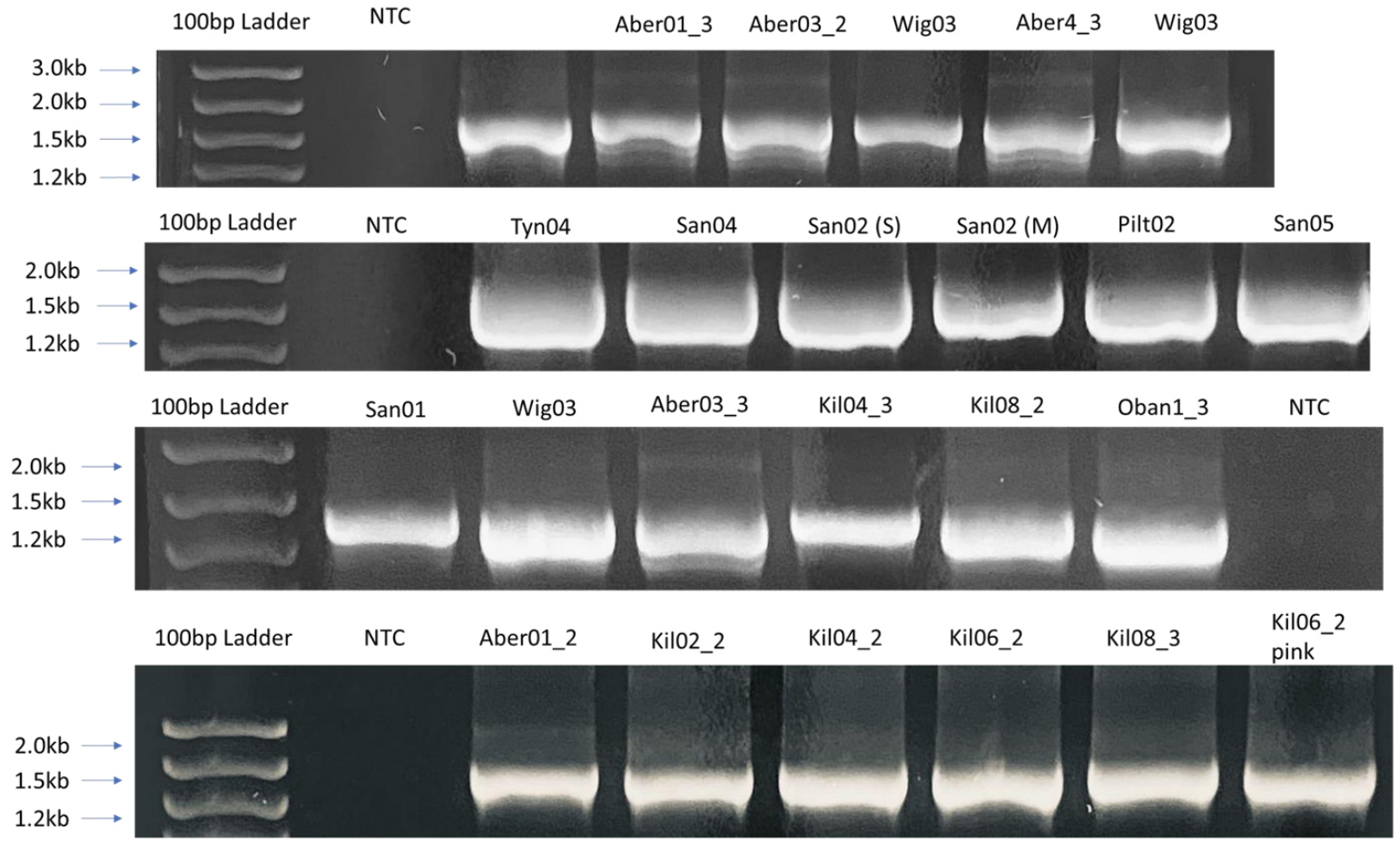

Figure 3.3 B. Two percent agarose gel electrophoresis showing results of polymerase chain reaction for the detection of 18s rDNA gene expression in isolated Thraustochytrids. Each gel contains a 100bp ladder and NTC lane contains a non-template control.

Table 3.2. Nucleotide BLAST results for each isolated thraustochytrids with the closest NCBI database match, length of 18S used for match generation (F- Full, P- Partial), Identity match generated by the database and the E value which the shows the probability that match was made at random.

Isolate	Closest NCBI database match	Length of 18S amplified	Identity match with database (%)	E value
Aber01_2	*Ulkenia visurgensis*	F	95.56	0.0
Kil02_2 (P)	*Thraustochytrium striatum*	P	98.43	0.0
Kil06_2	*Thraustochytrium kinnei*	F	98.00	0.0
Kil08_3	*Thraustochytrium kinnei*	P	99.43	0.0
Kil06_2 (P)	*Thraustochytrium striatum*	P	98.03	0.0
Kil04_2	*Thraustochytrium kinnei*	F	97.54	0.0
San-02 (S)	*Ulkenia visurgensis*	F	98.96	0.0
San-02 (M)	*Thraustochytrium kinnei*	F	96.76	0.0
San-01	*Aurantiochytrium sp*	F	96.98	0.0
San04	*Thraustochytrium kinnei*	P	98.88	0.0
Wig03	*Ulkenia visurgensis*	P	98.73	0.0
San05	*Thraustochytrium kinnei*	P	99.76	0.0
Aber04_3	*Ulkenia visurgensis*	F	95.84	0.0
Aber03_2	*Ulkenia visurgensis*	F	95.71	0.0
Aber03_3	*Ulkenia visurgensis*	F	95.47	0.0
Kil04_3	*Thraustochytrium kinnei*	P	97.55	0.0
Kil08_2	*Thraustochytrium kinnei*	P	99.36	0.0
Oban1_3	*Thraustochytrium kinnei*	P	99.39	0.0
Tyn_04	*Thraustochytrium kinnei*	P	99.72	0.0
Pilt02	*Thraustochytrium kinnei*	P	99.85	0.0

3.3 Stationary phase determination

To determine the stationary phase, the OD600 recordings of selected isolates were plotted over 7 days (**Figure 3.4**). Thraustochytrids reached their maximum growth between day 3 and day 6 with a sudden drop in cell density within the 24h period. One isolate, namely Aber01_2 showed no growth in the liquid media and therefore, it could not be included in the graph. Oban1_3, Tyn04, Kil06_2, Kil08_2, and San04 which were closely related to a strain of *Thraustochytrium kinnei* reached their highest biomass on day 4 with exception of Pilt02 which growth peaked on day 3 from inoculation. Kil2_2 (P) which is related to the strains of *Thraustochytrium stratium* had the longest growth phase of all isolates and reached densities surpassing 2A which is equivalent to the absorption of over 99% of the UV light passing through the 1mL of the measured sample. Its growth peaked on day 6 and it reached the highest biomass of all thraustochytrids tested. Wig03 and Aber04_3 which are strains of *Ulkenia visurgensis* reached the maximal growth on day 6. The biomass gain of San02 (*Ulkenia visurgensis*) and San01(Aurantiochytrium sp) over 7 days period was very low and did not produce distinguishable phases.

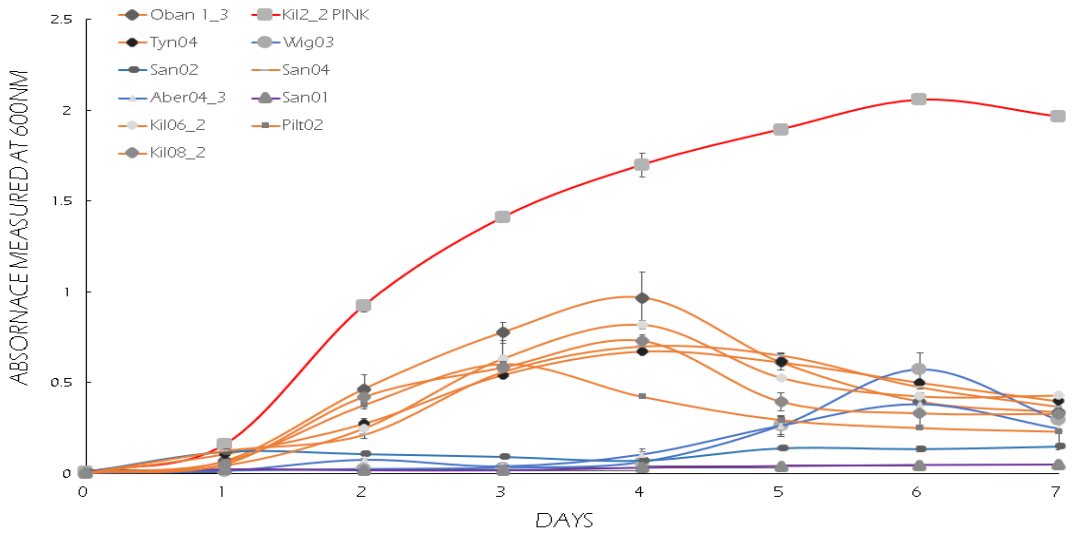

Figure 3.4 Growth curves of isolated Thraustochytrids in 2% glucose media, generated from OD600 absorbance data. The growth patterns in a modified GYP media containing 2% glucose were investigated for each isolate by daily measurement of OD600 and plotting it over the period of 7 days. The exponential phase can be clearly distinguished for each Thraustochytrid tested with a rapid drop in OD600 indicating the onset of a death phase. The curves's colours correspond to their taxonomic identification: red as *Thraustochytrium stratium*; Orange is *Thraustochytrium kinnei*; Blue is *Ulkenia visurgensis*; Purple is for *Aurantiochytrium sp*. The readings were taken in triplicate and standard diviation (SD) is expressed as vertical error bar for each day point.

3.4 Total lipid quantification

The standard curve prepared with known concentrations of Canola oil produced a line equation of y = 168.97x - 0.1565 with an R^2 = 0.9739 (Figure 3.5). The equation was used to calculate the total lipids content of isolated thraustochytrids from the absorbance data. The highest lipids content was obtained from Tynin04 which is estimated to be 7.2% of its biomass. The rest of the thraustochytrids showed lipid content between 3%-4% with Kil2_2 (P) at 5.35% of dry biomass.

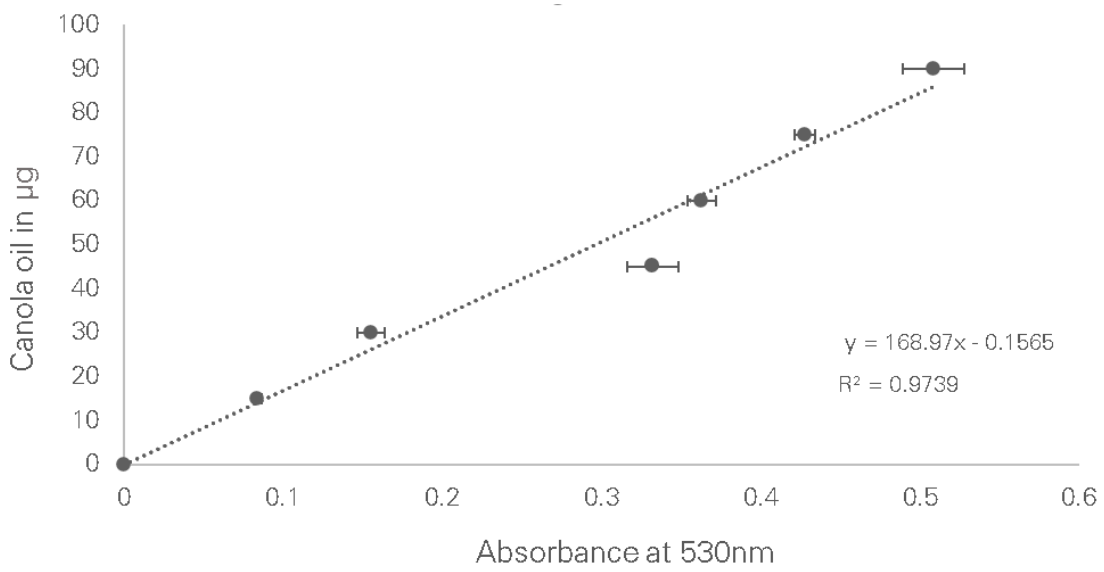

Figure 3.5. A standard curve showing a correlation between increasing Canola oil concentration and absorbance of phosphovanillin dye at 530nm.
The readings are expressed as mean value ± standard diviation (n=3). Line equation and R^2 value were generated by Excel software.

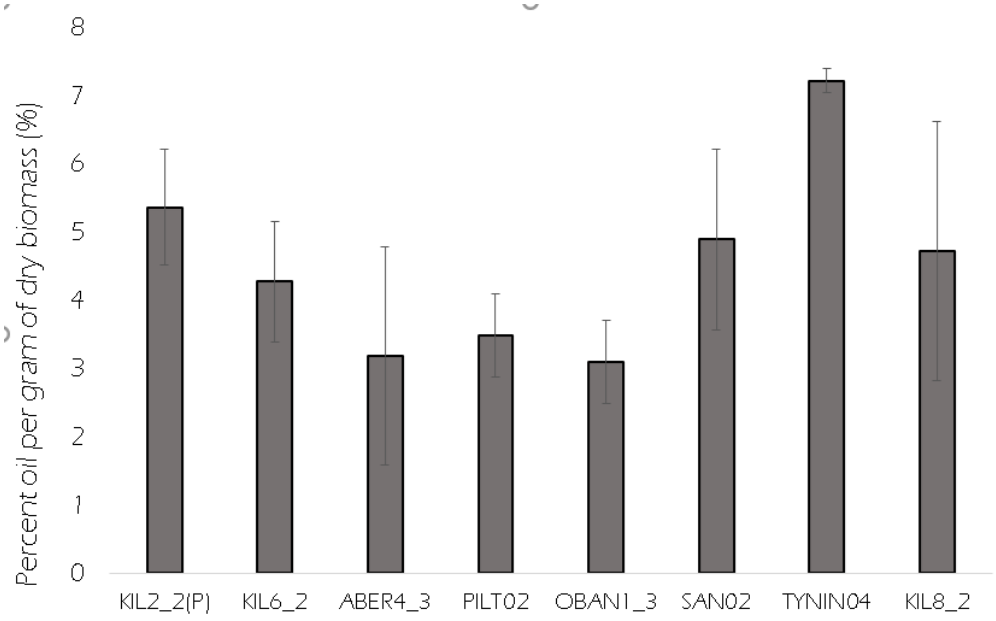

Figure 3.6. A bar graph showing percent of total fatty acids per gram of biomass for tested thraustochytrids.
The aborbance readings were taken in triplicate and the previously generated line equation was used to obtain the total lipid values. Bars represent mean value ± standard diviation (n=3).

3.5 Extracellular enzymes screening

Solid agar plates containing a specific enzyme-substrate were investigated for the presence of a clear halo surrounding the inoculation spot. Enzyme activity was determined by measuring the radius of the halo (**Figure 3.7 & Figure 3.8**). Only four thraustochytrids were able to digest the starch from the solid media (**Figure 3.7a**). Kil2_2(P) identified as a closely related to a strain of *Thraustochytrium striatum* showed the highest amylase activity, followed by Wig03, San02(G), and Aber01_2 corresponding to *Ulkenia visurgensis* strains. Low to medium chitinase activity was measured for Tynin04 (*Thraustochytrium kinnei*), San02(G), Wig03, and Aber01_2 in contrast to Kil2_2(P) which showed a high concentration of the enzyme (**Figure 3.7b**). Conversely, four isolates San02(S), Tynin04, Wig03, Aber01_2 showed a very low activity of cellulase on the plates as opposed to Kil2_2(P) which cellulase activity was the highest of all thraustochytrid strains (**Figure 3.7c**). In the case of lecithinase enzyme, the highest activity was recorded for San04 and Oban1_3 which were both identified as closely related to *Thraustochytrium kinnei* strains(**Figure 3.7d**). All thraustochytrid strains tested positive for caseinase presence with the highest enzyme activity shown by San04 and Wig03 (**Figure 3.7f**) and lowest caseinase for Aber04_3. However, there was no visible enzymatic activity on plates containing lignin in tested thraustochytrids and positive control organisms.

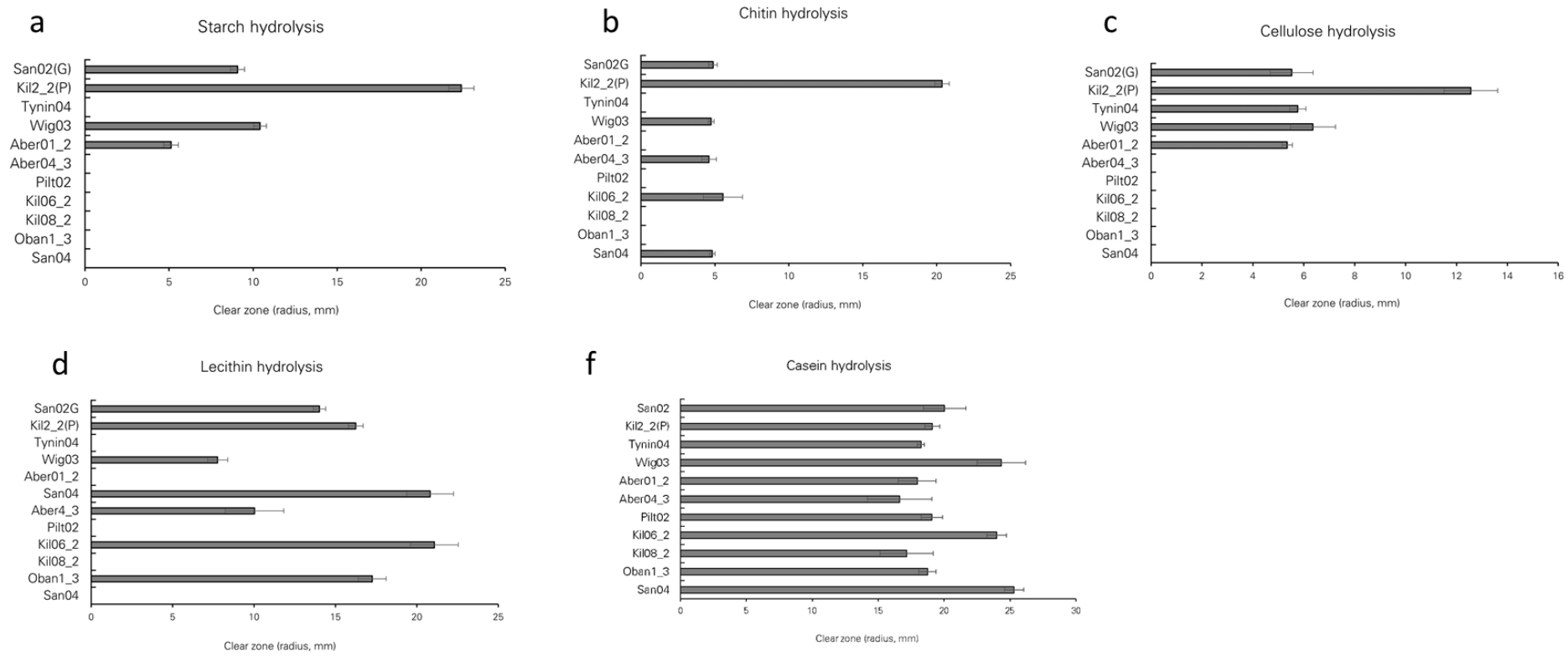

Figure 3.7 Enzyme activity produced by eleven strains of Thraustochytrids as observed by the zone of clearance.

a- Starch hydrolysis, b- Chitin hydrolysis, c-Cellulose hydrolysis, d- Lecithin hydrolysis, f- Casein hydrolysis. The zones of clearance were expressed as the radius of the halo in mm. Bars represent mean value ± standard diviation (n=3).

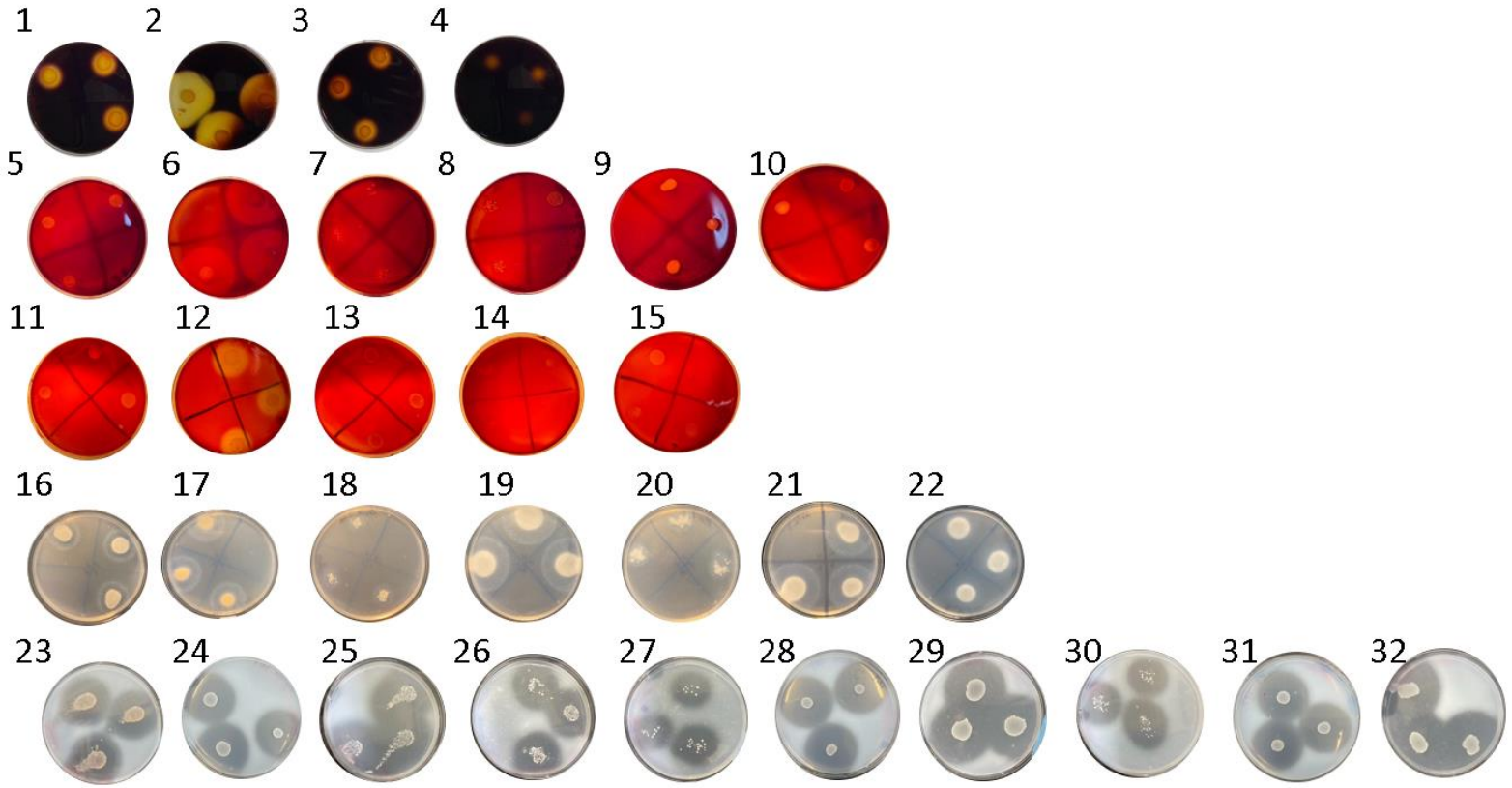

Figure 3.8 Zones of clearance indicating digestion of the substrate for agar plates containing:
Starch (1-4): **1**- San02(G), **2**- Kil2_2(P), **3**-Wig03, **4**-Aber01_2, 5; **Chitin (5-10): 5**- San02(G), **6**- Kil2-2(P), **7**- Wig03, **8**- Aber04_3, **9**-Kil06_2, **10**-San04; **Cellulase (11-15): 11**- San02(G), **12**-Kil2_2(P), **13**- Tynin04, **14**- Wig03, **15**- Aber01_2; **Egg yolk (16-22): 16**- San02(G), **17**- Kil2_2(P), **18**-Wig03, **19**- San04, **20**- Aber4_3, **21**- Kil06_2, **22**- Oban01; **Casein (23-32): 23**- San02(G), **24**- Kil2_2(P), **25**- Tynin04, **26**-Wig03, **27**- Aber01_2, **28**-Aber04_3, **29**- Pilt02, **30**- Kil06_2, **31**- Kil08_2, **32**- Oban1. The clear zone around inoculation spot suggest degradation of the substrate and is indicative of production of digestive enzymes. Each plate was inoculated in triplicate with one spot inoculation with clear media as a negative control.

3.6 Total protein content

Standard curve prepared with known concentrations of albumin protein from bovine serum produced a line equation of y = 7651x - 0.0331 with an R^2 = 0.9399 (**Figure 3.8**). The equation was used to calculate protein concentration in mg/mL (weight/volume of media) of isolated thraustochytrids from the absorbance data (**Figure 3.9**). The protein concentration in total cell biomass varied significantly and could not be successfully quantified with the standard curve for all the isolates. The highest protein concentration was measured in Oban1_3 isolate at 0.606 mg/mL which was closely followed by 0.601 mg/mL and 0.479mg/mL for Pilt03 and Tynin04, respectively. San01 absorbance results were outside the standard curve sensitivity level and therefore could not be quantified. In terms of, protein detected in the broth, only three isolates produced and secreted proteins that could be detected with this method. The highest concentration was measured for Kil2_2(P) with 0.299mg/mL which is significantly higher than the concentration of protein detected in the cell extract (P-Value = 0.003). However, San04 and Tynin04 protein concentrations in the media broth were significantly lower than in the cell extract with differences between 0.0155mg/mL in the broth and 0.271mg/mL in the cell extract for San04 (P-value = 0.000) and 0.0033mg/mL compared to 0.5340mg/mL for Tynin04 (P-value = 0.001).

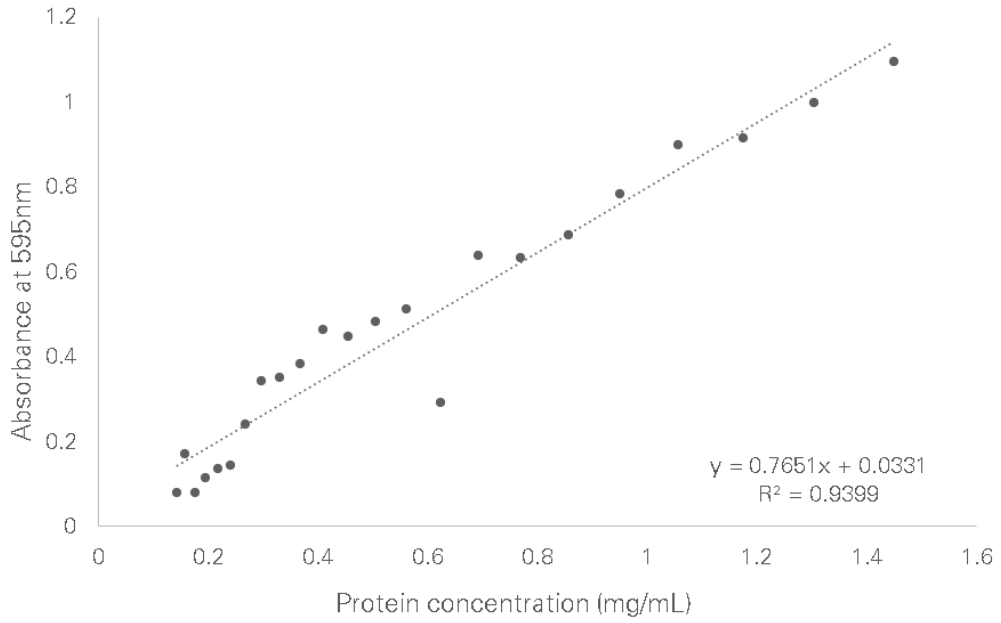

Figure 3.8. A standard curve showing a correlation between increasing albumin concentration and absorbance of Bradford dye at 595nm.
The readings are expressed as mean value ± of absorbance readings (n=3). Line equation and R^2 value were generated by Excel software.

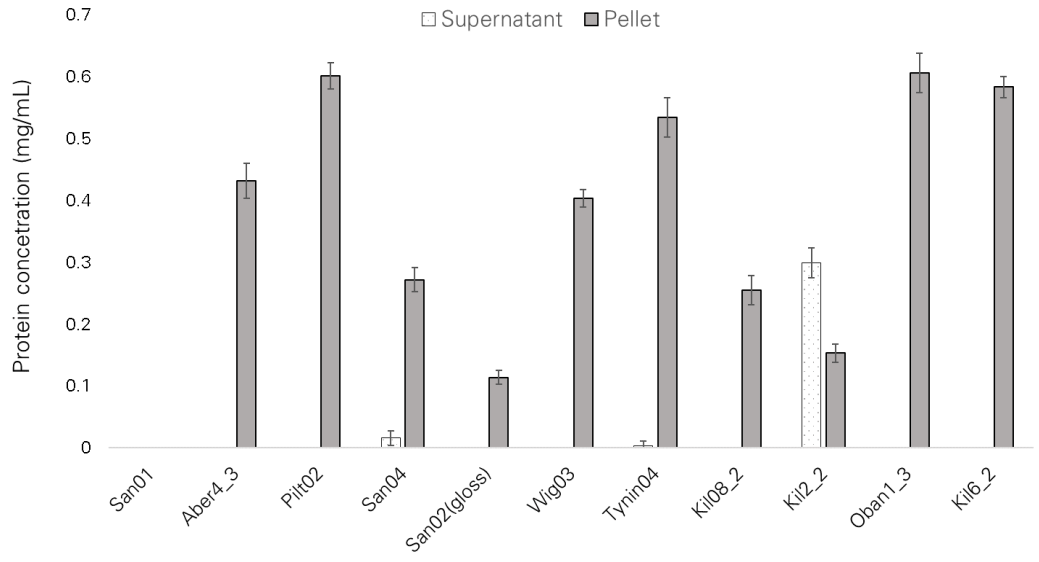

Figure 3.9. A bar graph showing protein concentration in mg/mL (weight per volume of media) of total protein extracted from the cell biomass and media supernatant in tested Thraustochytrid isolates.
The aborbance readings were taken in triplicate and the previously generated line equation was used for total protein estimation. The bars represent mean value ± standard diviation (n=3).

3.7 Emulsification assay

Only one thraustochytrid strain Oban1_3 produced an emulsification layer after vigorous mixing with 99% hexadecane (**Figure 3.10**). The emulsification layer measured at 10 minutes and 24h and was equal to 3mm and 3mm, respectively. No emulsification layer was observed for a negative control (DM-mod media).

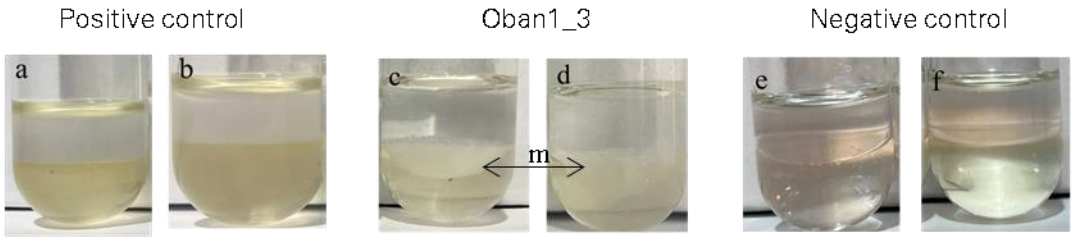

Figure 3.10. The results of emulsification test.
Layers separated after 10 minutes **(a, c, e**) and 24 hours (**b, d, f**) from mechanical distruption for positive control, Oban 1_3 and negative control samples. The emulsification layer was marked with "m". The absence of the meniscus between the top and the bottom phase indicates a reduced surface tension due to the presence of a surfactant.

3.8 Antimicrobial screening

After the incubation period, the agar plates with bacteria and yeast were inspected for the formation of halo around the discs containing thraustochytrid extracts. In this experiment, none of the screened thraustochytrids displayed antimicrobial activity in response to bacteria and fungi used in the study. As shown in **Figure 3.11(f- o),** the bacteria and fungi grew within the proximity of the discs without a sign of any inhibitory reaction. Moreover, the growth of *Lactobacillus Plantarum* on the solid media was not affected by the presence of 10mg of Gentamicin which served as a positive control in the study (**Figure 3.11d**). Similarly, *Saccharomyces cerevisiae* growth on the positive control plate was not affected by the presence of 1.5mg/mL of nystatin (**Figure 3.11c**). Furthermore, due to poor growth, plates containing *E.coli* failed to form a uniform layer on the solid media and therefore, the results remain inconclusive *(***Figure 3.11 g, l***)*.

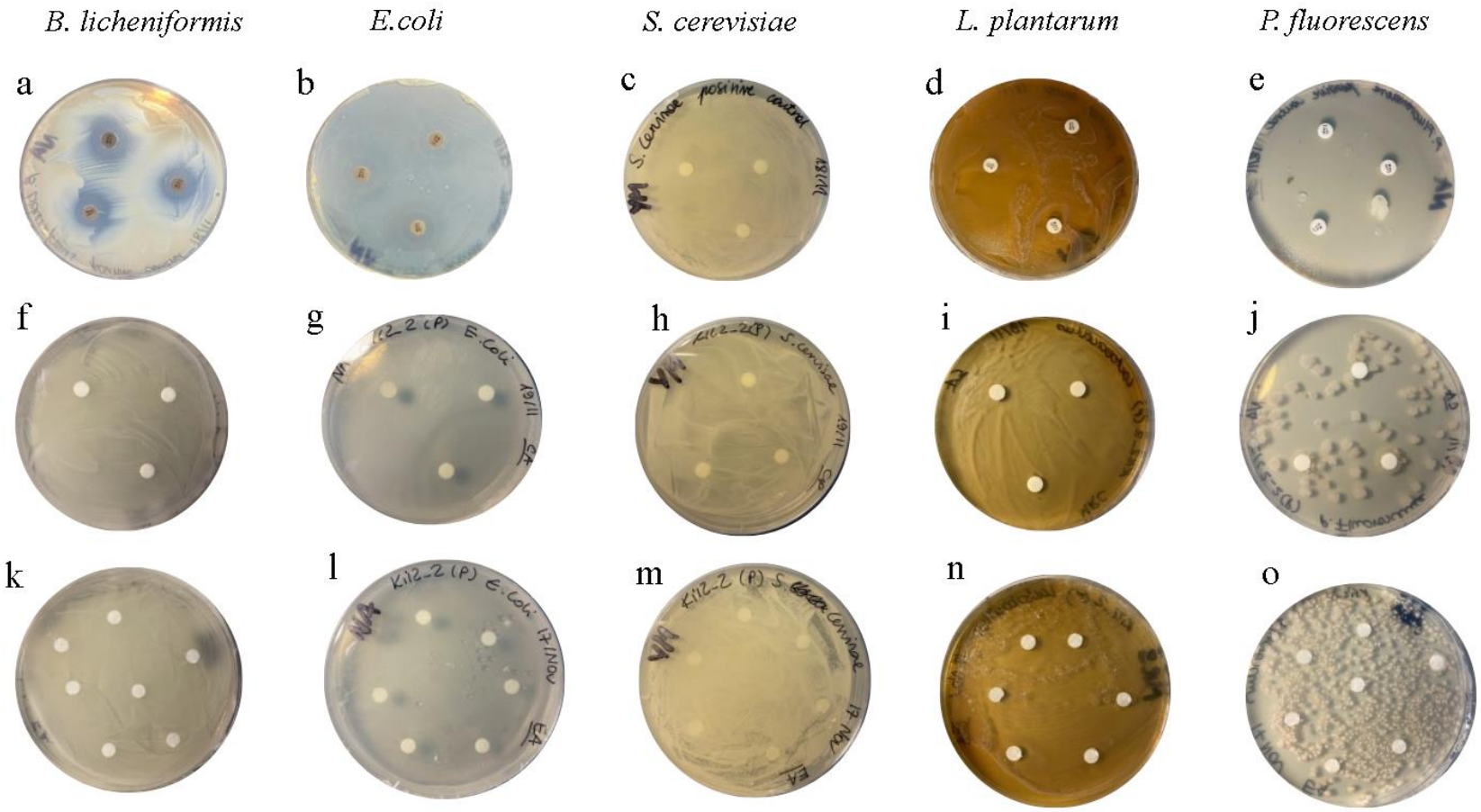

Figure 3.11. Agar plates showing growth of bacteria and fungi on plates containing positive control and Kil2_2(P) extracts.
Each disc contains:(a-d): 10 µg of Gentamicin; **(e)**: 1.5 µg of nystatin; **(f-j):** 30uL of Kil2_2(P) cell extract and **(k-o):** 30uL of Kil2_2(P) broth extract. The inhibitory activity is indicated by the transparent halo around the disc **(a-b)**. Absence of solid layer on the agar indicates poor growth of the microorganism **(g, l, e)**

Chapter 4- Discussion

Overall, the results indicate that thraustochytrids are abundant in the Scottish coastal waters and have the potential for use in biotechnological applications due to the production of valuable enzymes and biosurfactants.

The coastal saltmarsh ecosystem was proven to be adequate for thraustochytrid sampling likely due to the tidal flows that continuously flood the area delivering sediments and nutrients to the marsh. A higher number of thraustochytrids isolated between the warmer months of April to June indicates that their growth correlates with the seasonal changes in microbial biomass and nutrient availability that occurs in saltmarsh ecosystems [125]. Furthermore, the high abundance of thraustochytrids in the marine sediment supports their prominent role in benthic habitats where they are believed to participate in sedimentary organic decomposition [34]. However, a high number of other detritivores particularly soil bacteria, make their isolation from sediment significantly challenging even in presence of antibiotics and antifungals in the baiting media. Oil contamination seems to have a significant effect on the thraustochytrid numbers since none were found at the saltmarshes located near oil refinery despite studies showing their ability to sustain growth in presence of hydrocarbons [127]. A similar trend was observed in the areas where salinity was lower than 10ppt. Most thraustochytrids were isolated from areas where salinity varied between 23.6 to 32.5 ppt which can be explained by their obligate requirement for Na+ ions [126]. This is in line with the findings of Lee-Chang (2012) [54], who did not find any thraustochytrids in waters with salinity varying between 0.1–7 ppt. In terms of isolation method, the modified pine pollen baiting method developed in this study proved to be successful for the isolation of thraustochytrids, particularly those deriving from animal and plant detritus. However, since the success of pollen baiting depends largely on the ability of thraustochytrids to penetrate and extract nutrients from the exterior wall of pine pollen, it favors strains with well-developed ectoplasmic nets and a high concentration of hydrolytic enzymes. To account for other thraustochytrids, the method could be improved by direct plating of organic matter on highly nutrient media or by inoculating solid media with serially diluted seawater samples. In terms of media composition, the modified MC-BHB media developed by Rosa et al., (2011) [52] provides sufficient nutrients to sustain the growth of thraustochytrids on the solid

media. Indeed, high concentrations of various nitrogen sources have been shown to support the growth of thraustochytrids by providing a high nitrogen-to-carbon ratio necessary for proteins and *de-novo* cell synthesis [119], [128]. However, as mentioned before, the isolation of pollen-baited thraustochytrids from the sediment samples was largely unsuccessful due to the high load of bacteria and other microorganisms. Therefore, future isolation studies could benefit from supplementing the media with more wide-spectrum antibiotics to suppress bacterial growth.

Thraustochytrids are ubiquitous in the estuarine and marine habitats throughout the world. The results indicate the relative abundance of *Thraustochytrum* and *Ulkenia* genus in the coastal waters of Scotland. *Thraustochytrium kinnei* and *Ulkenia visurgensis* which strains have been identified as the major thraustochytrids inhabiting coastal waters of Scotland have also been isolated from other temperate and cold climates, as in southern Australia, Iceland, Argentina, and Antarctica [47], [48], [52], [54]. Moreover, the findings presented in this study identify the presence of *Thraustochytrium stratium* in the British coastal waters for the first time. Surprisingly, one species of *Aurantiochytrium* genus was also isolated even though members of this genus are more common in tropical and sub-tropical regions [129], [130]. Further analysis involving morphological study and phylogeny will be required to confirm their taxonomic classification.

Thraustochytrids follow four distinct phases of the growth curve: lag, exponential (log), stationary, and death [131]. Furthermore, it's been shown by multiple studies that these oleaginous tend to accumulate lipids during the stationary phase of their growth. In this study, the growth stages were defined by measuring the optical density of liquid media at each day of their culture. The results suggest that isolated thraustochytrids enter the death phase immediately after the exponential growth which is indicated by the sudden growth in cell density. This effect is likely due to rapid depletion of nutrients from the media or accumulation of cell-growth limiting by-products. The low cell densities recorded by spectrophotometric measurements are similar to trends in a study by Lee-Chang et al., (2014) [132] where slow growth of strains belonging to *Thraustochytrium* and *Ulkenia* genera were attributed to their inability to utilize glucose present in the media. Moreover, optical density reflects the concentration of cells in a liquid does not account for cell clumping which occurred in almost all of

thraustochytrid cultures and thus, may not be suitable for estimating their growth [133]. Therefore, future studies should include a dry cell weight (DCW) and may benefit from measuring glucose and pH fluctuations in media to account for nutrient utilization throughout the culture.

In terms of total fatty acids, it appears that lipids constitute a small part of the total cell mass of the isolated thraustochytrids under the culture conditions employed. These results are in contrast with other studies assessing total PUFAs with significantly higher yields for similar species [48], [51], [132], [135]. Based on the growth phase analysis, it is possible that because thraustochytrids entered the death phase immediately after the exponential phase they did not have the time to accumulate fatty acids which tend to occur during the stationary phase. In terms of limitations, the sulfo-phospho-vanillin assay is relatively novel for the detection of lipids in thrasutochytrids and its accuracy in these microorganisms has only been confirmed by one study [122]. Moreover, as in other colorimetric tests, the results are highly dependent on the reliability of the standard curve. Here, canola oil had a lipid composition that is significantly different than thraustochytrid oil. This is especially important since the basis of the assay is the spectrophotometric measurement of the color produced by the interaction of the sample's PUFA and multiple chemical reagents. Therefore, using a pure thraustochytrid oil would have produced a more reliable standard curve. Since there was no way to compare the output from the assay with another well-researched method such as gas chromatography (GC), the reliability of the results is questionable. Therefore, to fully assess the reliability of the results obtained in this study, a GC analysis would need to be conducted, which would also provide information about the proportion of any industrially valued DHA fractions and information about fatty acids fractions.

Due to their saprophytic nature, thraystochytrids have been reported to produce a variety of hydrolytic enzymes such as cellulase, protease, lipase, amylase, gelatinase, urease, and phosphatase [97], [98], [99], [100], [135]. The results confirm the ability of thraustochytrid to utilize complex organic matter as nutrient sources. In particular, Kil06_2 (P) identified in this study as closely related to a strain of *Thraustochytrium stratium* was shown to produce cellulase, chitinase, and amylase. This suggests that some thraustochytrids compete with bacteria for nutrients rather than feeding on left-over bacterioplankton supporting a dual role of thraustochytrids in the marine

ecosystem [27], [101]. In addition, the presence of chitinase indicates that they may be implicated in the nutritional enrichment of the coastal sediments by degrading refractory compounds [27]. However, this is in contrast with the notion that thraustochytrids are unable to degrade complex polymers, since herein the results suggest they possess enzymes enabling them to assimilate extracellular polysaccharides [98], [101]. Interestingly, thraustochytrids identified as strains belonging to two genera *Ulkenia* and *Thraustochytrium* hydrolyzed lecithin which assumes the presence of phospholipase. This indicates their ability to acquire nutrients from the surrounding lipids and potentially, may be implicated in the antimicrobial activity [99]. All strains showed high activity of protease which indicates their nutritional preferene for the degradation of proteins over complex carbohydrates such as from plants detritus. Thraustochytrids have not been widely analyzed for protein production, however, other studies showed that these strains could produce between 30- 50% DW of crude proteins [37], [132].In this study, the results suggest a very low protein content for all thraustochytrid screened. This disparity can be explained by the fact that herein the protein content is expressed in weight per volume of media as opposed to the weight per total biomass. In addition, future studies would benefit from including different carbon sources such as fructose, glucose, sucrose, and glycerol which were shown to significantly increase the protein production in Thraustochytrids [37]. Interestingly, when comparing protein content inside of the cells and surrounding media, the *Thraustochytrium stratium* appears to excrete most of its protein extracellularly. Because this strain has also been shown to produce the biggest variety of enzymes, the results suggest that *Thraustochytrium stratium* may play a key role in the degradation of organic compounds and may be more involved in organic matter circulation than other thraustochytrids. Nevertheless, since enzyme profiles tend to vary considerably between the member of the same species and genera, each strain may possess its own unique set of extracellular enzymes that allows it to preoccupy a specific niche in the coastal ecosystem.

Hitherto, biosurfactant activity has never been reported before for thraustochytrids. Here the results show for the first time the ability of a thraustochytrid, in this case the a strain shown as closely related to *Thraustochytrium kinnei* to produce emulsifying agents in the presence of hydrocarbon. By evolution, microorganisms have adapted to feed on water-immiscible materials via the production of surface-active products that help them to solubilize and absorb these materials. Estuarine systems are known for accumulating

particulate organic matter and contaminants including petroleum hydrocarbons, and as such it is possible that thraustochytrids may have acquired the ability to extract nutrients from these substances [136]. Furthermore, the presence of phospholipase in Oban1_3 means that it can acquire nutrients from the emulsified lipid phase. Biosurfactants offer countless applications in industry such as in bioremediation where they are capable of degrading hydrocarbon pollutants by increasing their bioavailability to other microorganisms [137]. This would considerably expand the current biotechnological application of thraustochytrids.

Since the habitat of thraustochytrids is rich in competing bacteria and fungi it was speculated that they may be capable of producing compounds with antimicrobial properties. However, extracts from all the thraustochytrid isolates in this study were found unable to inhibit the growth of the bacteria *Lactobacillus plantarum*, *Bacillus subtilis, Bacillus licheniformis, E.coli K12, Pseudomonas fluorescens*, and the fungus *Saccharomyces cerevisiae*. The results support previous claims that thraustochytrids are unable to directly affect microorganisms around them through the synthesis of antimicrobial compounds. It is also in contrast to a similar study by Kalidasan et al., (2015)[110] in which thraustochytrid extracts inhibited the growth of various pathogenic bacteria. However, the study by Kalidasan et al., (2015) [110] failed to specify the taxonomic identity of the thraustochytrid used in its experiments and therefore, it remains unclear whether these microorganisms are indeed capable of antimicrobial activity. The study herein had multiple limitations. Firstly positive control containing wide-spectrum antibiotic gentamicin failed to inhibit the growth of *Lactobacillus plantarum* which susceptibility to the antibiotic has been shown by previous studies [126]. Similarly, 1.5mg of nystatin failed to inhibit the growth of Saccharomyces cerevisiae despite claimed susceptibility [138]. The reason for this discrepency is largely unknown but could be due to the antimicrobial resistance of these particular strains of bacteria and fungi. Therefore, future studies should use a mixture of multiple antibiotics and antifungals of a wide spectrum of action to avoid these issues. Another limitation is the extraction procedure. It is possible that the bioactive compounds could have been degraded or lost during the process of solvent extraction or cell disruption, particularly if they were present at low concentrations. Therefore, a future studies may benefit from including a more direct method such as that employed by Lopes et Sangorrin (2010) [139] which involves direct streaking of tested

microorganism onto a bacterial lawn and observing the formation of a zone of inhibition. This, however, will require further optimization before it could be adapted for thraustochytrids including the establishment of optimal media and temperature that would allow the growth of both thraustochytrid and microorganism tested.

4.1 Conclusions and future work

The research aimed to isolate Thraustochytrids from Scottish coastlines and to screen them for the production of high-value products. Based on qualitative analysis of total fatty acids, hydrolytic enzymes, antimicrobial agents, protein content, and biosurfactants, it can be concluded that Scottish thraustochytrids have the potential to play a significant role in the biotechnological industry. The results indicate that two isolated thraustochytrids, namely Kil2_2(P) identified as closely related to *Thraustochytrium stratium* and Oban1_3 identified as closely related to *Thraustochytrium kinnei* are suitable candidates for hydrolytic enzymes and biosurfactants sectors, respectively. Overall, Scottish coastal waters were found to be abundant in thraustochytrids particularly during warmer months of late spring and summer. The modified method involving pollen baiting and successive dilutions was suitable for the isolation of thraustochytrids from sediments and organic detritus of Scottish coastal saltmarshes. As a result, twenty Thraustochytrids were isolated from which two genera seem to predominate, namely *Thraustochytrium* and *Ulkenia* with strains closely related to *Thraustochytrium kinnei*, *Thraustochytrium stratium*, and *Ulkenia visurgensis* as major species.

In addition, the use of spectrophotometric measurement of cell densities was proved to be sufficient in estimating general growth trends of isolated thraustochytrids, despite the lack of clearly distinguished stationary phase. In terms of semi-quantitive lipid estimation, it appears that lipids constitute a small part of the total cell mass of the isolated thraustochytrids. Sulfo-phospho-vanillin used in the study is a quick and cheap method for total lipids quantification however, its reliability needs further validation with other better-established methods such as GC. Additionally, a GC study would support the total lipids data since it would also include the fatty acids profiles of screened thraustochytrids thus, providing DHA and other PUFAs percentage content.

Similarly, Bradford staining proved to be a good pre-preliminary test for estimating protein content in both cells and media extracts despite generally low protein content in isolated thraustochytrids. Furthermore, enzyme digestion plates showed the ability of thraustochytrids to utilize a complex organic matter as nutrient sources through the production of various hydrolytic enzymes. Particularly, of all screened thraustochytrids Kil2_2 (P) was shown to be most promising due to its versatile enzymatic profile containing chitinase, cellulase, and amylase, protease, and lecithinase. In addition, pre-preliminary screening for biosurfactant activity identified Oban1_3 as a potential producer of biosurfactants. Unexpectedly, none of the screened thraustochytrids showed antimicrobial activity. This enforces the commonly accepted belief that these microorganisms rely on their unique enzymatic set to gain an advantage in the environment as opposed to directly affecting competing microorganisms around them through the release of antimicrobial compounds.

Further research is needed to fully elucidate the applicability of isolated thraustochytrids in the biotechnological industry. Particularly, in terms of biosurfactant capabilities of Oban1_3 strain. Since the results herein are preliminary further experiments such as taking surface tension measurement, purification of the biosurfactant will aid its characterization. In terms of enzymatic activity, the next step would likely involve extraction, purification, and characterization of the chitinase, cellulase, and amylase enzymes and comparing their catalytic power against other commercially available microbial enzymes. Furthermore, the presence of other commercially available enzymes should be also investigated including catalase, cyclodextrin glucanotransferase, dextranase, galactosidase, glucanase, hemicellulose, inulase, laccase, lipase triacylglycerol, lysozyme, or urease. Similarly, further research into lipids of isolated Thraustochytrids may benefit from optimizing culture conditions. This may likely involve adopting other carbon sources such as glycerol or incorporating cold-shock to induce lipid production. In addition, since the identification of stationary phase is crucial for optimizing lipid production in Thraustochytrids, future research should include dry cell weight (DCW), extend the time frame to account for slow-growing strains, and measure glucose and pH fluctuations in media to account for nutrient utilization throughout the culture. Lastly, utilizing established methods such as GC will provide more reliable results that can be more accurately compared with data from similar studies.

Overall, this study confirmed the relative abundance of thraustochytrids in Scottish coastal regions as well as established a reliable method of their isolation. The results herein also argue against an accepted notion that thraustochytrids were unable to assimilate complex carbohydrates by proving their capability to digest chitin, cellulose, and starch. Finally, for the first time, we show that thraustochytrids are capable of producing emulsifying agents in presence of hydrocarbon, thus expanding their current biotechnological application.

Chapter 5 -References

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