Studies on
Bacteriolytic Activities of
Marine Labyrinthulids

Md. Iftekharul Wahid

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The United Graduate School of Agricultural Sciences
Kagoshima University, Kagoshima, Japan

Contents

Abstract

Thirteen strains of labyrinthulids were isolated from the coastal area of Kagoshima Bay, Kagoshima Prefecture, Japan and from Batan Bay, Panay Island, the Philippines by using diatom double-layer agar plates. The cells of labyrinthulid isolates grew axenically on the bacterial dead cells and extract plus egg yolk agar medium (NSBEY agar). Three representative isolates were demonstrated to belong to the labyrinthulid phylogenetic group (LPG) based on 18S rDNA sequence analysis. In the hydrolysis test of labyrinthulid isolates, it was found that they could hydrolyse macromolecular compounds like starch. Ten enzymes were detected by using API-ZYM Kit in four representative isolates. Three enzymes including alkaline phosphatase, leucine arylamidase and valine arylamidase were found in higher level in API-ZYM tests. As examined their bacteriolytic activity, it was found that they could lyse only the dead cells of Gram-negative bacteria and not those of Gram-positive bacteria during incubation in both agar and liquid media. The optimum temperature range for their bacteriolysis was from 25 to 31^{o}C. Respiratory inhibitors such as sodium cyanide, dinitrophenol and sodium azide had repressed bacteriolysis activity but no impact on viability was found in *Labyrinthula* cells of a strain 00–Bat-05, Philippine isolate. Orange carotenoid pigment(s) was accumulated during stationary growth phase of strain 00-Bat-05, cultured in an L-shape tube containing a bacterial dead cell suspension and concomitantly rapid cell movement of developing zoospores was observed.

Leucine aminopeptidases (LAPs) from marine labyrinthulid strain 00-Bat-05 and thraustochytrid strain HR-3 cells were partially purified and characterized by enzymological properties. The optimum temperature of LAPs from both strains was 37^{o}C. The thermostability of 00-Bat-05 LAP was indicated by having 80% of maximum activity after heat treatment at 60^{o}C for 10 min, while LAP activity of strain HR-3 was completely inactivated at 60^{o}C. LAP acvtivities from both strains were high at near pH 8.0. Both LAPs were inhibited by 1,10-phenanthroline, *p*-chloromercuribenzoic acid (PCMB), bestatin and sodium dodecyl sulphate (SDS), suggesting that both are SH-aminopeptidase. Enzyme activity of LAP from strain 00-Bat-05 was stimulated by Co^{2+} and inhibited by Zn^{2+}, while that from HR-3 was inhibited by Co^{2+} and Zn^{2+}. LAP of 00-Bat-05 had a high specificity for L-leucine-*p*-nitroanilide but HR-3 enzyme showed relatively broad specificity for *p*-nitroanilide derivatives of L-amino acids. Partially purified LAP enzyme, from labyrinthulid sp. strain 00-Bat-05, showed the

bacteriolysis activity against the dead cells of *Vibrio parahaemolyticus* which phenomenon was also found by the cells of strain 00-Bat-05.

Chapter I

General Introduction

Part 1. Review of Literatures

A. Labyrinthulids and Thraustochytrids

General characteristics

Members of the phylum Labyrinthulomycota (labyrinthulids and thraustochytrids) are eukaryotic that produce heterokont zoospores (Figure I-1). The evolution of extraordinary diversity in eukaryotic organisms constituting the kindgom Protoctista is exemplified by Labyrinthulomycota, the slime nets. These type organisms produce globose or colonical structures associated with wall-less ectoplamic networks which absorb nutrients and attach the cells to surfaces. Their cell walls are composed of thin golgi-derived scales. The ectoplasmic networks, devoid of ribosomes and other cellular organelles, are produced by organelles at the cell surface termed sagenogens, also called sagenogenetosomes or bothrosomes. Reproductive cycles of labyrinthulids and thraustochytrids involve the formation of heterokont, biflagellate zoospores (Figure I-2) (Porter, 1990).

The Labyrinthulomycota phylum contains a single class, Labyrinthulea, and a single order, the Labyrinthulida, with two families, the Labyrinthulidae and the Thraustochytridiidae (Olive, 1975). Labyrinthulidae contains a single genus, *Labyrinthula*, with eight recognized species. The Thraustochytriidae includes seven genera and 30 species, half of which are in the genus *Thraustochytrium*. In the mycological literature these families are called the Labyrinthulaceae and the Thraustochytriaceae.

Grown in the laboratory, these saprotrophic to weakly parasitic organisms are found primarily in marine and estuarine environments, usually associated with benthic algae,

marine vascular plants, and detrital sediments. There are no reliable culture collections of these organisms and so, they must be obtained from nature, or researchers.

Labyrinthula was first observed by Cienkowski (1867) who described two species, *L. vitellina* and *L. macrocystis,* associated with intertidal algae in the Black Sea. On the other hand, the genus *Thraustochytrium* was first observed by Sparrow (1936), who described *T. prolferum* associated with benthic algae from Massachusetts, USA.

Significance

Labyrinthula was reported to be parasitic on a number of different marine vascular plant and marine algae (Raghukumar, 1986; 1987a and 1987b). Attention had been focused on *Labyrinthula* after the "wasting disease" epidemic of *Zostera marina* (eelgrass) in the early 1930s, which destroyed most of the vast subtidal stands of this vascular plant along the Atlantic coasts of North America and northern Europe. Recurrence of wasting disease was observed in the Mediterranean on 1966 (den Hartog *et al*., 1966) and in the New Hampshire, USA on 1984 (Short *et al*., 1986). *Labyrinthula zostera*, most well known species of labyrinthulids, is reported as the causative agent of wasting disease of eelgrass (Renn, 1936; Young, 1943; Muehlstein *et al*., 1988, 1991). Since larval stages of shellfish, such as oysters, scallops, and shrimp, depend on eelgrass as a nursery bed, the loss of these eelgrass stands had devastating effects on the seafood industry. Several decades elapsed before the eelgrass beds were completely re-established. Although no outbreak of the wasting disease has been reported in recent years, *Labyrinthula* is still commonly associated with eelgrass. It was proved that their infection pattern was genus-specific (Vergeer, 1994). The disease may have been the result of subtle changes in a combination of abiotic factors, including salinity, light

intensity, and temperature, which increased the susceptibility of *Zostera* to *Labyrinthula* (Pokorny, 1967). More recent and more surprising, however, is the sudden appearance of labyrinthulids causing rapid blight disease of cool-season grasses on golf course turf (Olsen *et al*., 2003). Although they have been known to inhabit some ponds and pools with a higher-than-average salinity, terrestrial forms of the disease previously had been extremely rare.

Ecology and habitats

Species of *Labyrinthula*, found in estuarine and near-shore marine habitats throughout the world, are associated with or isolated from organic detritus, macroalgae, diatoms and particularly, marine vascular plants, such as *Spartina, Zostera* and *Thallassia*. One species of *Labyrinthula* is claimed to be parasitic on the fresh water alga *Vaucheria sessilis,* and another has been found associated with the roots and root hairs of trees in sandy soils irrigated with low salinity (4.3 ppt) water (Aschner, 1958). *Labyrinthula* spp. have also been isolated from inland saline soils (Amon, 1978) and from the spore cases of vesicular-arbuscular mycorrhizal fungi from the face of barrier sand dunes (Koske, 1981).

In laboratory cultures, cells of *Labyrinthula* readily attack a variety of vascular plant and algal tissues. They penetrate the cell walls and decompose the cellular contents. The ectoplasmic networks of *Labyrinthula* are also capable of decomposing many different microorganisms. Bacteria, yeast, fungal hyphae, diatoms, filamentous algae, and even thraustochytrids in laboratory cultures can serve as sources of nutrition for *Labyrinthula*. Whether or not these organisms are food for *Labyrinthula* in nature is not known. Healthy algae and marine grasses, from which *Labyrinthula* may be isolated, do not

contain *Labyrinthula* cells within their cells or tissues. *Labyrinthula* is probably not necrotrophic but more likely feeds on epibiotic microorganisms and decomposing plant and algal material. Species of *Labyrinthula* are most reliably isolated from moribund or drift leaves of marine vascular plants and pieces of filamentous and thalloid macroalgae (Porter, 1990).

In mangrove brackish water environments, *Labyrinthula* spp. live on submerged fallen mangrove leaves and parasitizes fungal hyphae, which are decomposing the leaves. The stramenopiles, fungi and *Labyrinthula*, play important role in mangrove ecosystem by fixing leaf nutrients in a form of hyphae or cells and converting the leaf to a nutritious food for leaf consuming animal (Nakagiri, 2001).

Thraustochytrids species are also cosmopolitan; they have been taken from estuarine and marine habitats throughout the world. Thraustochytrids have been found to be associated with various organic detritus materials through the use of pine-pollen baiting techniques and other enrichment cultures. Thraustochytrids are generally isolated from decomposing algal and plant material, as well as from sediments, although they also have been taken from offshore water samples. Members of the thraustochytrids are able to grow in culture on a variety of plant and animal derived natural substrates (Perkins, 1973).

Quantitative methods for determining the prevalence of thraustochytrids in nature, using a serial dilution and pine-pollen baiting technique, were pioneered by a German scientist, Gaertner. The number of propagules (thalli or spores) found in offshore waters or clean estuarine waters ranges from 10 to 100 spores per liter of water. But thraustochytrids are rarely isolated from ocean sediments from below 1500 meters (Gaertner, 1982).

Characterization

The family Labyrinthuliidae contains a single genus, *Labyrinthula*, with eight or nine recognized species. *Labyrinthula* is classified as producing heterokont on the basis of the morphology of the zoospores (Perkins and Amon, 1969) and an ongoing study into the phylogenetic position of *Labyrinthula*, *Thraustochytrium* and *Labyrinthuloides* spp. has determined to form a monophylectic group within the Heterokonta (Honda, 2001).

The single genus, *Labyrinthula*, is characterized by spindle-shaped cells completely enclosed by the hyaline filaments of the ectoplasmic network. They exhibit gliding cell motility within the ectoplasmic networks. This trophic phase consists of a colony of a variable number of cells which can increase indefinitely by mitotic cell division within the common ectoplasmic network. As their unusual nature, *Labyrinthula* spp. were reported to be capable of decomposing various microbial living cells including yeast, fungal hyphae, microalgae and bacteria in laboratory condition and found to prefer Gram-negative bacteria as food. (Yokochi *et al*., 2002; Perkins and Amon, 1969; Perkins, 1973). The gliding motility of the cells, which at times is as fast as 100 μm/min, is thought to be related to the presence of a calcium-dependent contractile system of actinlike proteins in the ectoplasmic network (Nakatsuji and Bell, 1980).

Labyrinthula can produce long-chain polyunsaturated fatty acids (PUFA) or functional lipid in the cells (Yokochi, 2001 and Kumon, 2002) and decosahexaenoic acid (DHA) was found to account for 58-68 % of total fatty acids derived from whole-cell lipid in labyrinthulids (Sakata *et al*., 2000). Decosahexaenoic acid is one of the most widespread species of polyunsaturated fatty acids (PUFA), which plays key roles in brain, visual and nervous systems in human.

The family Thraustochytriidae contains 30 species in seven genera. Among them, genus *Thraustochytrium* and *Schizochytrium* deserve more attention in aquaculture because they accumulate high concentrations of polyunsaturated fatty acids such as docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA) which are essential nutrients for many fish and crustaceans (Singh and Ward, 1997; Nakahara *et al*., 1996; Sakata *et al*., 2000; Miller *et al*., 2007). Thraustochytrids are used practically as a feed for zooplankton such as rotifers and *Artemia* (Hayashi, 2002) because of improving the nutritional value of the food animals.

B. Leucine Aminopeptidase (LAP)

1. Animal LAP

Name and history

Linderstrøm-Lang (1929) reported the presence of an enzyme in erepsin (extracts of pig intestinal mucosa) that cleaved leucylglycine about 20 times faster than it cleaved glycylglycine. This peptidase was provisionally named dipeptidase II, but later referred to as aminoleucylpeptidase in a Danish review article (Holter, 1979). Johnson *et al*. (1936) partially purified the enzyme, and then called leucylpeptidase. Although it is now clear that leucine aminopeptidase (LAP) hydrolyzes a wide variety of peptides and amides, this name has persisted. The activation of LAP by magnesium and manganese was shown by Johnson *et al*. (1936) and Berger and Johnson (1939). Studies by Spackman *et al*. (1955) and Smith and Spackman (1955) on purified LAP from swine kidney revealed many chemical and physical properties of the enzyme. The zinc-metalloenzyme nature of LAP was determined by Himmelhoch (1969).

LAP was also among the seven crystalline proteins first obtained in Sumner's laboratory (Dounce and Allen, 1988). However, the identity of these protein crystals was unknown at that time and the protein was named the 'football protein', based on the resemblance of the crystals to an American football. Fifty years later the football protein was finally identified as beef liver LAP (Dounce and Allen, 1987). LAP was the first dizinc enzyme for which a crystal structure at atomic resolution was determined (Burley *et al*., 1990). Other names for the enzyme are cytosol aminopeptidase and peptidase S.

Activity and specificity

Pig kidney LAP is maximally active between pH 9 and 9.5 and cleaves a variety of aminoacid amides, dipeptides and other compounds (Spackman *et al*., 1955; Smith and Spackman, 1955; Delange and Smith, 1971). All tested substances with an N-terminal L-amino acid (or glycine) are hydrolyzed; however, compounds that have L-leucine residues in the N-terminal position are the preferred substrates. Peptides having proline in P1′ (Xaa-Pro-) are not cleaved by LAP. Esters are also substrates of the enzyme, although they are cleaved at about 10% of the rate of the corresponding amides.

For an assay of LAP activity in vitro the release of the products of L-leucine-*p*-nitroanilide or of L-leucine-*β*-naphthylamide is followed spectrophotometrically or fluorometrically. The transformation of the hydrolytic product into a diazo complex has been used to enhance the accuracy of the assay in complex matrices (Goldbaryg *et al*., 1959). A more recent assay is based on two-photon excited fluorescence detection on a microchip (Zugel *et al*., 2000).

LAP is inhibited by the naturally occurring inhibitors bestatin and amastatin in cattle lens enzyme and pig kidney LAP, respectively (Rich *et al*., 1984; Taylor *et al*., 1993). Compared with a true peptide substrate these inhibitors have an extra carbon atom that bears a hydroxyl group. Bestatin also inhibits other aminopeptidases. The natural inhibitor shows low toxicity in humans and has been used to explore immune response, retardation of tumor invasion and growth, and degradation of peptides and proteins (Scornik and Botbol, 2001). L-leucylthiol (Chan, 1983) is also strong inhibitor of LAP.

Structural chemistry

Cattle lens LAP is a homohexamer of 324 kDa molecular mass. Its primary structure was determined by chemical sequencing (Cuypers *et al*., 1982) and for cattle kidney LAP, from the cDNA sequence (Wallner *et al*., 1993). The monomer has a mixed $\alpha + \beta$ structure and consists of an N-terminal domain (160 amino acid residues) and a catalytic C-terminal domain (327 amino acid residues) (Burley *et al*., 1991, 1992). LAP does not cleave longer peptides or proteins (Kim *et al*., 1974).

The two metal ions in LAP work different exchange behavior: the site 1 metal ion is readily exchanged against other metal ions, including Mn^{2+}, Mg^{2+} and Co^{2+} (Carpenter and Vahl,1973; Thompson and Carpenter, 1976a, b). Exchange of Zn^{2+} against Mn^{2+} or Mg^{2+} in site 1 activates LAP. Zn^{2+} is much more tightly bound and can only be replaced by Co^{2+} (Allen *et al*., 1983). The unambiguous identification of the two metal-binding sites in LAP was achieved by a structure determination of the Mg-Zn enzyme, in which Mg^{2+} occupied the readily exchangeable site 1 (Kim and Lipscomb, 1993).

Preparation

Procedures for the purification of LAP from cattle lens (Allen *et al*., 1983) and pig kidney to homogeneity have been published. Cytosolic pig kidney LAP is also available from commercial sources (Sigma). This commercial preparation can be purified as described by Van Wart and Lin (1981).

Biological aspects

LAP has different tissue-specific physiological roles in the processing or degradation of peptides. Human LAP has been shown to catalyze postproteasomal trimming of the N-terminus of antigenic peptides for presentation on major histocompatibility complex (MHC) class I molecules (Beninga *et al*., 1998). Altered LAP activity has been implicated in certain pathological conditions such as human eye cataracts (Taylor *et al*., 1984).

LAPs are also found in bacteria: *Escherichia coli* aminopeptidase A (PepA) shows significant homology to cattle lens LAP, including a conserved active-site structure (Stirling *et al*., 1989; Sträter *et al*., 1999). The homology between the LAPs from different kingdoms (animals, plants, bacteria) is about 30–40% for the whole protein and higher in the catalytic C-terminal domain. The active-site residues involved in metal binding and catalysis are conserved between all LAPs characterized so far.

Distinguishing features

Other aminopeptidases have substrate specificities similar to those of LAP and have to be distinguished by their molecular properties. The bacterial aminopeptidases from *Vibrio proteolyticus* and *Streptomyces griseus* also contain a dizinc center and have a

preference for peptides with an N-terminal leucine residue. However, these peptidases are smaller monomeric proteins and the active-site structures are different from LAP. Another group of zinc aminopeptidases that should not be confused with LAP consists of the membrane alanyl aminopeptidases which are also sometimes named leucine aminopeptidase. These have a subunit molecular mass around 110 kDa.

2. Plant LAP

Name and history

Linderstrøm-Lang and Sato (1929) described a leucylpeptidase activity (peptidase II) in extracts of germinated barley seeds with an alkaline pH optima. In 1939, Berger and Johnson showed that the leucylpeptidases from barley, cabbage and spinach were activated by Mn^{2+} and Mg^{2+}, like the porcine leucyl aminopeptidase. Leucine aminopeptidases (LAPs) from barley and kidney bean were subsequently purified and biochemically characterized (Sopanen and Mikola, 1975; Mikkonen, 1992). Both the barley and kidney bean LAPs have multimeric structures similar to those of the animal LAPs.

In the early 1990s, the cDNAs with high sequence identity to the bovine LAP were identified in *Arabidopsis* (Bartling and Weiler, 1992), tomato (Pautot *et al*., 1993) and potato (Hildmann *et al*., 1992). A tomato LAP antiserum identified four classes of LAP-related proteins in wounded tomato leaves: the 66 and 77 kDa LAP-like, the 55 kDa neutral LAP (LAP-N) and 55 kDa LAP-A proteins (Gu *et al*., 1996b). Most plants express the LAP-like proteins and a single LAP form similar to the tomato LAP-N (Chao *et al*., 2000; Tu *et al*., 2003). Only a subset of solanaceous plants express the wound-induced 55 kDa LAP-A (Herbers *et al.*, 1994; Chao *et al*., 2000).

Activity and specificity

LAPs have been purified from barley and kidney bean seeds and after overexpression in *Escherichia coli*. All plant LAPs are thermostable metallopeptidases with alkaline pH optima (pH 8.5–9.5). Both the barley and kidney bean seed LAPs are stimulated by Mg^{2+} and Mn^{2+} (Sopanen and Mikola, 1975; Mikkonen, 1992) and, like the animal LAPs, bestatin abolishes the kidney bean LAP activity.

The tomato mature LAP-A and His_6-LAP-A enzymes were purified to homogeneity from *E. coli* extracts and were extensively characterized in parallel with the porcine LAP and *E. coli* PepA (Gu *et al*., 1999; Gu and Walling, 2000, 2002). The tomato LAP-A1 metallopeptidase is stimulated by Mn^{2+} and Mg^{2+} and inhibited by amastatin and bestatin (Gu *et al*., 1999). *In vitro* the tomato LAP-A1 enzyme hydrolyzes a putative precursor of the bioactive peptide systemin, but alone this enzyme does not catabolize systemin (Gu and Walling, 2000). The *in vivo* substrates of plant LAPs are currently not known.

Structural chemistry

The native plant LAPs are 320–360 kDa (Sopanen and Mikola, 1975; Mikkonen, 1992; Bartling and Nosek, 1994; Gu *et al*., 1996b). The subunit compositions of the native barley and *Arabidopsis* LAPs are unknown. The wound-induced LAP of tomato is a homohexamer of 55 kDa LAP-A subunits (Gu *et al*., 1996b; Gu and Walling, 2000). In contrast, the kidney bean LAP is a heterohexamer with protomers of 58 and 66 kDa (Mikkonen, 1992). The putative Zn^{2+}-binding and catalytic residues identified in the bovine LAP and *E. coli* PepA are conserved in all plant LAPs (Gu and Walling, 2002). The ability of a plant LAP protomer to bind zinc ions has not been evaluated.

Preparation

The purification of the native LAPs from barley and kidney bean has been described (Sopanen and Mikola, 1975; Mikkonen, 1992). The mature tomato LAP-A1 was overexpressed in *E. coli* and purified to apparent homogeneity (Gu *et al*., 1999). The wild-type tomato His_6-LAP-A1 and 31 mutant His_6-LAP-A1 proteins overexpressed in *E. coli* were purified to homogeneity using Ni-nitrilotriacetic acid resin columns (Gu and Walling, 2000, 2002).

Biological aspects

Most animals, microbes and plants express a single class of LAP polypeptide. These LAPs are ubiquitous in the plant kingdom, detected in all plant organs and do not respond to environmental or hormonal signals. The *Arabidopsis* LAP and tomato LAP-N are representative of this class (Bartling and Nosek, 1994; Chao *et al*., 2000; Tu *et al.*, 2003).

A subset of solanaceous plants (tomato, potato and nightshade) express a second wound-inducible form of LAP (LAP-A) with subunits having acidic pIs (5.6–5.9). Two tomato genes (*LapA1* and *LapA2*) (Gu *et al*., 1996a; Ruiz-Rivero and Prat, 1998) encode *LapA* RNAs, which are abundant in floral organs and fruit. *LapA* genes are not expressed during plant vegetative development unless challenged by pathogens, chewing insects, wounding, water-deficit stress or salinity (Pautot *et al.*, 1993; Chao *et al*., 2000). Tomato *LapA* genes are transcriptionally controlled and promoters have been studied (Ruiz-Rivero and Prat, 1998; Chao *et al*., 1999, 2000). The potato *Lap* genes are similarly regulated (Hildmann *et al*., 1992). In contrast, LAP-A proteins are abundant in both healthy and wounded nightshade leaves (Chao *et al*., 2000). Antisense *Lap* potato

and tomato plants do not exhibit development abnormalities or altered responses to pathogen infection or chewing insects (Herbers *et al*., 1994; Pautot *et al*., 2001). *Lap* expression is not completely abolished in these lines; therefore roles of LAP-A in plant defense remain to be elucidated.

Related peptidases

Alkaline aminopeptidase activities with features similar to LAPs are detected in crude tissue extracts from pine nut (Guitton and De Belsunce, 1963), Scots pine (Salmia and Mikola, 1975), wheat (Mounfield, 1935), peanut (Mikola, 1976), and were partially purified from squash (Ashton & Dahmen, 1967). However, the hexameric nature of these enzymes was not determined. There are many other plant enzymes called leucine aminopeptidase (Walling and Gu, 1996); however, the majority of these enzymes are monomeric with the ability to hydrolyze leucyl chromogenic substrates.

A number of organisms with photosynthetic abilities, but lying lower than plants on the evolutionary scale, have sequences very similar to that of plant LAP. For the most part these are known only as protein sequences and their proteolytic properties have not been examined. These include: *Nostoc punctiforme, Nostoc* sp. PCC7120, *Prochlorococcus marinus, Synechococcus* sp. PCC942, *Synechoccus* sp. WH8102, *Synechocystis* sp., *Synechocystis* sp. PCC680, *Thermosynechoccus elongatus* and *Trichodesmium erythraeum.*

3. Bacteria LAP

Name and history

A heat-stable aminopeptidase with similar biochemical properties to eukaryotic

leucine aminopeptidase (LAP) was purified to homogeneity from *Escherichia coli* K-12 by Vogt (1970) and named aminopeptidase I. An enzyme with similar properties was also isolated from the insoluble fraction of *E. coli* B by Matheson and co-workers, and referred to as the basic or ribosomal aminopeptidase (Dick *et al*., 1970; Tsai and Matheson, 1965). Subsequently, Miller and co-workers found an enzyme similar to aminopeptidase I in cell extracts of *Salmonella typhimurium*, and called it peptidase A (PepA) (Miller and Mackinnon, 1974). *S. typhimurium* mutants lacking PepA were identified and the *pepA* gene was mapped (Miller, 1975; Miller and Mackinnon, 1974). A similar enzyme, and *pepA* mutants, were then identified in *E. coli*, and recognized to be equivalent to aminopeptidase I (Miller and Schwartz, 1978). PepA is frequently referred to as aminopeptidase A by these workers. PepA homologs have now been characterized from a number of other bacterial species.

More than 60 bacterial genes with a high level of similarity to *E. coli* PepA have been sequenced as part of ongoing genome sequencing projects, though at least 12 of these are more closely related to PepB than they are to PepA. Members of the monomeric di-zinc M28 family of peptidases are also referred to as bacterial leucyl aminopeptidases but are not related to aminopeptidases PepA. The PepA-like, non-PepB, proteins will therefore be treated here as leucyl aminopeptidase PepA or aminopeptidase PepA.

Activity and specificity

The closely related *E. coli* and *S. typhimurium* PepA aminopeptidases are the best characterized of the bacterial enzymes. They exhibit similar substrate specificity and properties to mammalian leucyl aminopeptidases. PepA cleaves the N-terminal L-amino

acid from a broad range of peptide substrates, with a preference for methionine or leucine as the N-terminal amino acid (Matheson *et al*., 1970; Miller and Mackinnon, 1974; Vogt, 1970). Tripeptides are cleaved faster than dipeptides and peptides as long as six amino acids in length can be substrates (Vogt, 1970). Studies with *S. typhimurium* peptidase mutants indicate that PepA is unable to cleave substrates where the second residue is proline (Miller and Green, 1983). PepA is heat stable and is active over a broad range of pH (from 7.0 to 10.5) and ionic strength (20–500 mM KCl or NaCl) (Vogt, 1970). PepA is a metalloenzyme, requiring Mn^{2+} or Mg^{2+} for full activity, and is inhibited by EDTA. Some peptidase activity has been observed with Co^{2+}, Ni^{2+} and Cd^{2+} ions, but not with Ca^{2+}, Cu^{2+} or Fe^{2+} (Vogt, 1970). PepA is completely inhibited by Zn^{2+} (Vogt, 1970; Stirling *et al*., 1989). Bestatin, a slow binding inhibitor of eukaryotic LAPs, inhibits the *P. aeruginosa* homolog of PepA (Woolwine *et al*., 2001).

A convenient assay for aminopeptidase PepA activity uses L-leucine *p*-nitroanilide as substrate. The increase of absorbance at 405 nm as *p*-nitroaniline is released has been used to measure aminopeptidase PepA activity present in heat-treated bacterial crude extracts as well as to quantitate the activity of the purified enzyme (Stirling *et al*., 1989; Wood *et al*., 1993; Woolwine *et al*., 2001). Proline *p*-nitroanilide is also a substrate for *E. coli* PepA (Little *et al.*, 1994). Efficient cleavage of L-leucine *p*-nitroanilide by PepA requires Mn^{2+} rather than Mg^{2+} (Stirling *et al*., 1989). Vogt (1970) used small peptides as substrates for PepA and measured the extent of reaction using the ninhydrin method.

Preparation

E. coli PepA has been purified from crude extracts (Vogt, 1970) and from *E. coli* overexpressing PepA from a recombinant plasmid (McCulloch *et al.*, 1994; Stirling *et*

al., 1989). Purification takes advantage of the heat stability of PepA and its insolubility at low ionic strength. *E. coli* PepA can be further purified by ion-exchange chromatography, hydrophobic-interaction chromatography or gel filtration (Alén *et al*., 1997; Vogt, 1970). *P. aeruginosa* PhpA has also been overexpressed in *E. coli* and partially purified (Woolwine *et al*., 2001).

Biological aspects

PepA is one of three broad-range aminopeptidases, with overlapping substrate specificity in *E. coli* and *S. typhimurium* (the others being PepN and PepB). These three aminopeptidases, together with the dipeptidase PepD, can hydrolyze a variety of peptides supplied in the growth medium, allowing their use as amino acid sources (Miller and Mackinnon, 1974; Little *et al*., 1994). PepA also cleaves and activates several peptide toxins. PepA is one of two peptidases in *S. typhimurium* capable of activating albomycin, though albomycin is not cleaved by *E. coli* PepA (Braun *et al.*, 1983). PepA appears to be the only peptidase capable of cleaving alafosfalin in *S. typhimurium* (Gibson *et al.*, 1984). PepA is one of the major peptidases that hydrolyzes glutathione-derived Cys-Gly in the cysteine salvage pathway of *E. coli* (Suzuki *et al.*, 2001). PepA also functions in the final steps of protein turnover during starvation (Yen *et al*., 1980), and in the degradation of abnormal proteins (Miller and Green, 1981). Presumably PepA has similar functions in other bacterial species.

Bacterial leucyl aminopeptidases have a number of other functions, at least some of which are unrelated to their peptidase activity. These functions include: participation in Xer site-specific recombination, regulation of the *carAB* operon in *E. coli* and *S. typhimurium*, control of alginate expression in *P. aeruginosa*, and pH regulation of

virulence genes in *V. cholerae*. Production of cholera toxin and other virulence proteins is regulated by both temperature and pH in *V. cholerae*. Disruption of *pepA* in *V. cholerae* leads to elevated expression of virulence genes at the non-inducing pH (Behari *et al.*, 2001). It seems likely that PepA functions as a transcriptional repressor in the regulatory cascade that controls virulence gene expression in *V. cholerae*.

Distinguishing features

PepA aminopeptidases are distinguished by their large size and hexameric structure. PepA is the only L-leucine *p*-nitroanilide hydrolyzing enzyme that survives heat treatment at 70°C in crude extracts of *E. coli* (Vogt, 1970). Although purified PepB is heat stable, it does not survive heat treatment in crude extracts (Mathew *et al.*, 2000). Unlike their di-zinc eukaryotic counterparts, the bacterial leucyl aminopeptidases are inhibited by Zn^{2+} (Stirling *et al*., 1989; Vogt, 1970). *E. coli* and *S. typhimurium* PepA proteins are insoluble and precipitate at low ionic strength (Miller and Mackinnon, 1974; Vogt, 1970).

Related peptidases

Bacterial leucyl aminopeptidases are related to the eukaryotic leucyl aminopeptidases found in both plants and animals. They are also related to the PepB aminopeptidases. Peptidase My from *Mycoplasma salivarium* appears to consist of two different protein chains, one of which bears substantial amino acid similarity with other leucyl aminopeptidases (Shibata *et al*., 1995).

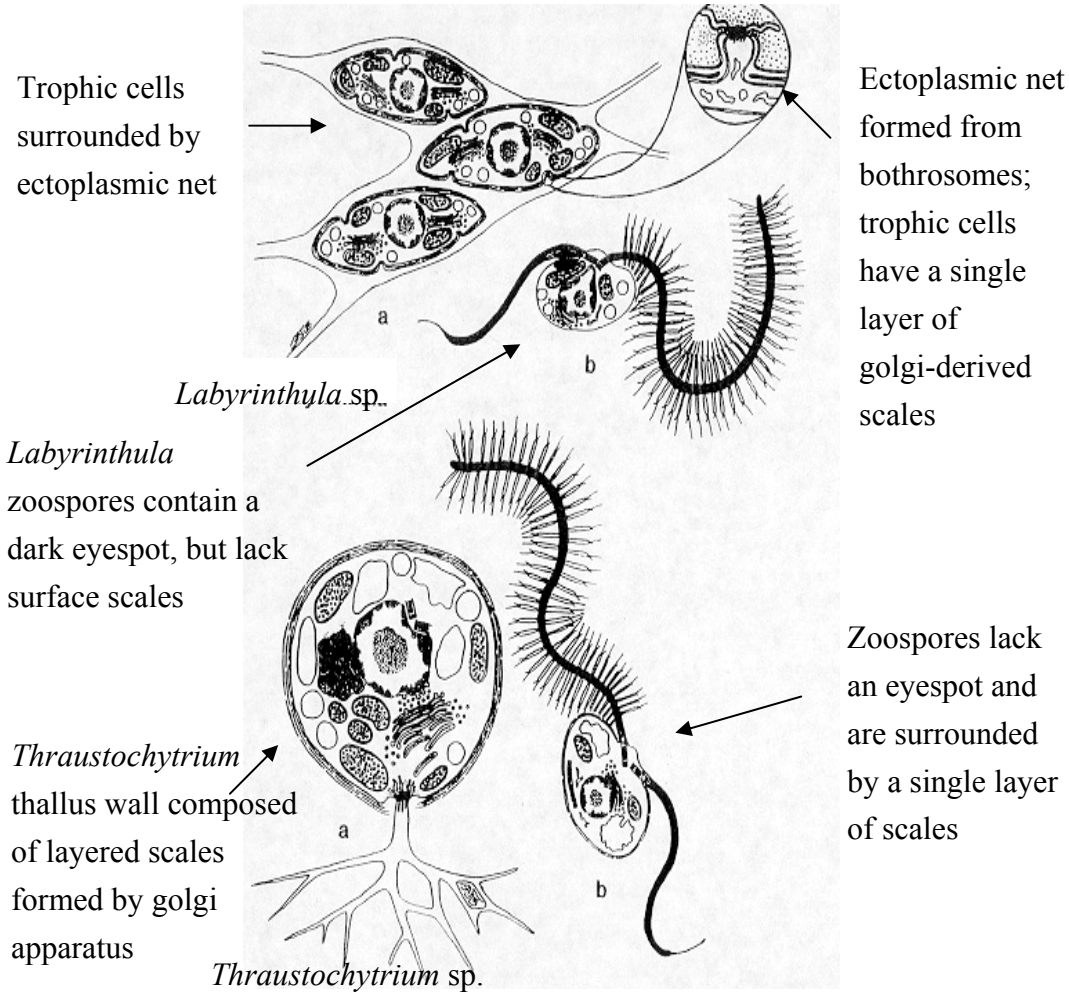


Figure I-1. Diagrammatic cell (a) and zoospore (b) difference between labyrinthulids and thraustochytrids (Porter, 1990).

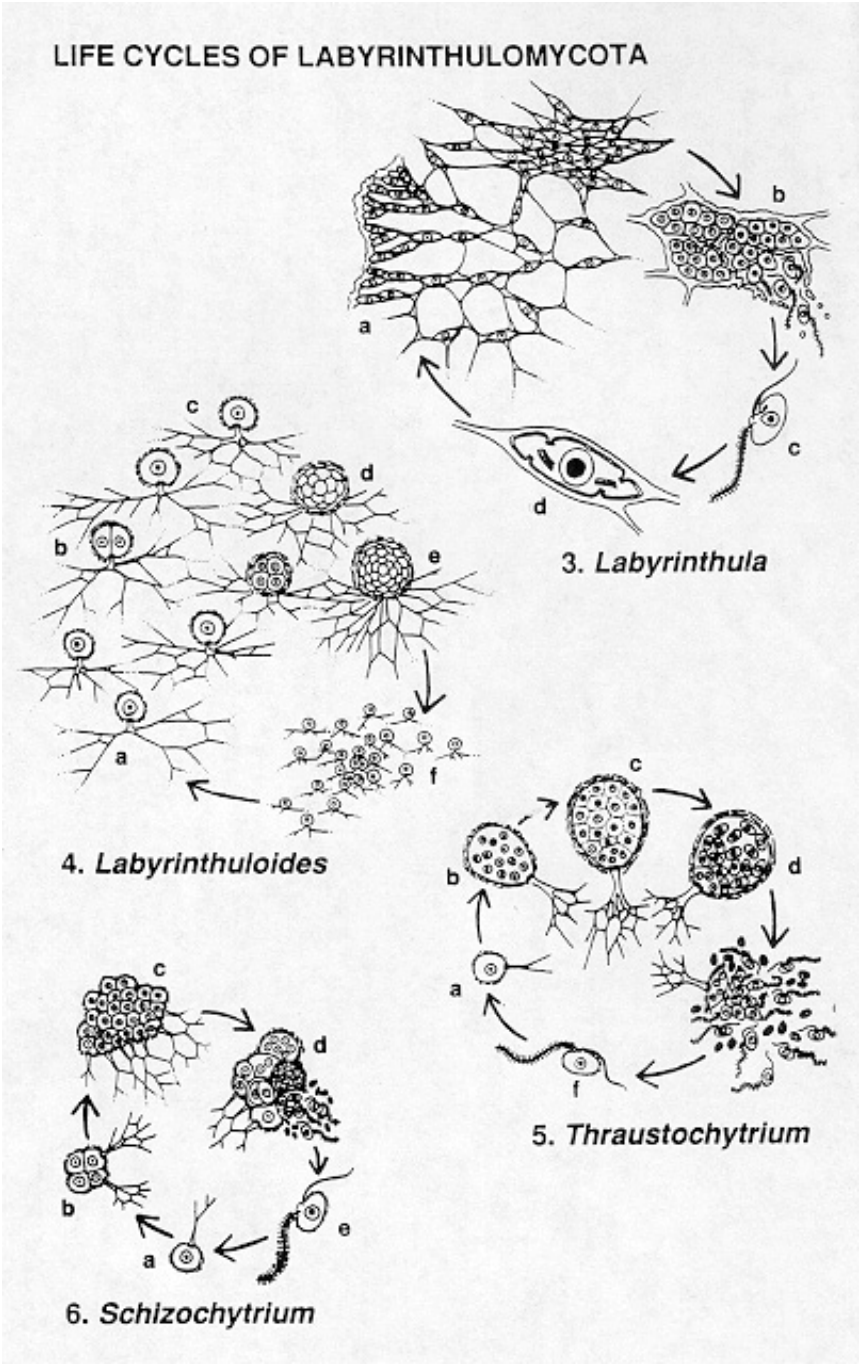


Figure I-2. Life cycles of labyrinthulids (3) and thraustochytrids (5 and 6) in a schematic presentation (Porter, 1990).

Part 2. Research Objectives

Labyrinthula was first observed by Cienkowski (1867) who described two species, *Labyrinthula vitellina* and *L. macrocystis,* associated with intertidal algae in the Black Sea. *Labyrinthula* has attracted attention through the wasting disease of eelgrass (*Zostera marina*) in the 1930s, which destroyed most of the vast subtidal stands of this vascular plant. Labyrinthulids are biflagellate protists and heterotroph and inhabit various plant materials in marine environments by digesting microorganisms, such as bacteria, yeasts, molds and micro algae, which are living their surface. *Labyrinthula* spp. play an important role in mangrove ecosystem by fixing plant nutrients in a form of hyphae or cells of the organisms and converting them to a nutritious food for plant consuming animals (Nakagiri, 2001) and play the peculiar roles in marine food web systems (Porter, 1972; Cavalier-Smith, 1993 and Honda *et al*., 1999). More recent and more surprising, however, is the sudden appearance of labyrinthulids causing rapid blight disease of cool-season grasses on golf course turf (Olsen *et al*., 2003). Although they have been known to inhabit some ponds and pools with a higher-than-average salinity, terrestrial forms of the disease previously had been extremely rare.

To knowing about this unusual organism, *Labyrinthula* and its bacteriolytic activity, the objectives of this study are

- To isolate of *Labyrinthula* strains from marine environments
- To examine the growth response of *Labyrinthula* on various medium and culture methods
- To examine the bacteriolytic activity of isolated labyrinthulid strains under various conditions including temperature and inhibitors

- To isolate and characterize of bacteriolytic agents (enzymes) and to compare with other closely family (thraustochytrids) member's enzyme.

Chapter II

Bacteriolytic Activity and

Growth of Marine Isolates of Labyrinthulids

on Bacterial Dead Cells

Introduction

Labyrinthulids and thraustochytrids are slime mold-like protists and distributed widely in marine environments such as estuarine and near-shore habitats. In brackish water mangrove area, labyrinthulids are known to live on submerged mangrove leaves or parasitize fungal mycelia which are decomposing plant leaves. Furthermore, *Labyrinthula zosterae*, most well-known species of labyrinthulids, was reported as the causative agent of wasting disease of eelgrass, *Zostera marina* (Muehlstein *et al*., 1988; 1991, Short *et al*., 1987; Young, 1943). Thus labyrinthulids play an important role in mangrove and seaweed ecosystems by converting plant materials to a form of their vegetative cells or ectoplasmic network and providing them for the upper layer animals of the food web as nutritious foods (Armstrong *et al*., 2000; Yokochi *et al*., 2002; Perkins and Amon, 1969).

The ectoplasmic network of labyrinthulids can digest bacteria, yeast and different microorganisms (Perkins and Amon, 1969; Porter, 1972). Bacteria, yeast, fungal hyphae, diatoms, filamentous algae, and even thraustrochytrids in laboratory cultures can serve as sources of nutrition for *Labyrinthula* spp. Whether or not these microorganisms are foods for *Labyrinthula* spp. in nature is not well known. Healthy algae and marine grasses are not known to contain *Labyrinthula* cells within their tissues, although *Labyrinthula* spp. are isolated from their whole bodies. *Labyrinthula* spp. are more likely feed on epibiotic microorganisms as well as to be necrotrophic to decompose plant and algal materials (Porter, 1972, 1990; Pokorny, 1967). If *Labyrinthula* spp. live on a wide variety of microbes as food sources in natural environments, they have an important role on microbial loops in the food web (Porter, 1990; Klie *et al*., 1968; Sakata et al., 1995, 1996).

The enigmatic characteristics of labyrinthulids in the physiology and morphology have been left unexplored, mainly due to the difficulty of cultivating and maintaining the axenic culture. So far labyrinthulid cells have been cultured and maintained on agar media containing living yeast, *Rhodotorula rubra* (Klie *et al*., 1968) or diatom cells *Chaetoceros ceratosporum* (Sakata *et al*., 1995, 1996) as food organisms. In this chapter of the study, the author examined axenic culture methods using bacterial dead cells in order to obtain large amount of growing cells of marine *Labyrinthula* spp., and simultaneously determined their biological activities including bacteriolysis, growth, pigment formation and sporulation during incubation with bacterial dead cells.

Materials and Methods

Isolation of marine labyrinthulids

1. Sampling sites

Samples were collected from seawater, seaweeds, macroalgae and mangrove leaves in the coastal area of Kagoshima Bay (130°30-50'E, 31°10-45'N), Kagoshima Prefecture, Japan and from submerge mangrove leaves of Batan Bay (122°80'E, 11°50'N), Panay Island of the Philippines as shown in Table II-1. The seawater and seaweeds samples were taken from culture of kuruma shrimp (*Penaeus japonicus*), yellowtail (*Seriola quinqueradiata)*, flatfish (*Paralichthys olivaceus*) and different places of the Kagoshima Bay. Submerge mangrove leaves (fallen) and soils were taken from under the mangrove trees. The samplings were also conducted in MBC aquaculture farm in Kokubu, the coastal area of Aira, Takoyama, Sesegushi mangrove

project area and the aquaculture experimental station of Kagoshima University in Kamoike, Kagoshima city.

2. Media composition

2.1. Algal medium

ES (Provasoli's enrichment seawater) medium, 1000 mL

NSW	1000 mL
ESP	40 mL
0.1%-Na_2SiO_3	5 mL

a) Preparation of ES medium:

Liquid medium: Five (5) mL of 0.1%-Na_2SiO_3 was added in 1000 mL of filtrate NSW (Natural Sea Water). NSW and 40 mL of ESP solution were sterilized in an autoclave at 0.8 psi, 120^oC for 20 min. After that, ESP solution was added.

Solid medium: For normal and soft agar medium 1.5% and 0.8% agar were used in liquid medium, respectively.

ESP enrichment solution (100 mL)

$NaNO_3$	350 mg
Na_2-β-glycerophosphoric acid	50 mg
Fe (EDTA)	2.5 mg
Vitamin B_{12}	10 μg
Biotin	5 μg
Thiamin HCl	500 μg
Tris	500 mg
PII metal solution	25 mL
Distilled water	100 mL
pH	7.8

b) Preparation of ESP solution:

$NaNO_3$ 350.0 mg, Tris 500.0 mg and Na_2-β-glycerophosphoric acid 50.0 mg were taken in a 100 mL flask. Fe (EDTA) 25.0 mg was dissolved in 10 mL DW (distilled water) and 1 mL of this solution was added into the flask. Vitamin B_{12} 10.0 mg and biotin 5.0 mg were dissolved in 10 mL of DW and from this solution 1 mL was diluted in 9 mL of DW and 50.0 mg of thiamin HCl was added to this solution. From the thiamin solution, 1 mL was taken out and diluted in 9 mL of DW (vitamin mixture) and then, 1 mL of that vitamin mixture was added to the 100 mL flask and 25.0 mL of PII metal solution was also added. Finally, DW was added to make 100 mL volume.

PII metal solution (100 mL)

$Na_2EDTA{\cdot}2H_2O$	110.7 mg
$FeCl_2{\cdot}6H_2O$	4.8 mg
H_3BO_3	114.3 mg
$MnCl_2{\cdot}4H_2O$	14.4 mg
$ZnCl_2$	1.0 mg
$CoCl_2{\cdot}6H_2O$	0.4 mg
Distilled water	100 mL

c) Preparation of PII metal solution:

$Na_2EDTA{\cdot}2H_2O$ 110.7 mg and H_3BO_3 114.3 mg were taken in a 100 mL flask. $FeCl_2{\cdot}6H_2O$ 48.0 mg and $MnCl_2{\cdot}4H_2O$ 144.0 mg were separately dissolved in each 10 mL of DW and 1 mL from each of solutions were taken and added to 100 mL flask. $ZnCl_2$ 100.0 mg and $CoCl_2{\cdot}6H_2O$ 40.0 mg were separately diluted in each 10 mL of DW and 1 mL from each diluted solution was mixed with 9 mL of DW. Then, 1 mL from each of mixture solution was added into the 100 mL flask and finally DW was added to make up 100 mL volume of the flask.

Silicon solution (100 mL)

Na_2SiO_3	0.1 g
Distilled water	100 mL

d) Preparation of Si solution:

Na_2SiO_3, 0.1 g was dissolved in 100 mL of distilled water and stocked after autoclave sterilization.

2.2. Bacterial medium

Z-CII agar medium (1000 mL)

Polypeptone	5.0 g
Yeast extract	1.0 g
Distilled water	625 mL
2ASW (Artificial Sea Water)	375 mL
Agar powder (1.5 %)	15.0 g
pH	7.6

a) Preparation of Z-CII medium:

The reagents, except agar powder, were mixed together in a beaker and the pH was adjusted using 4% NaOH solution. Then agar was added and dissolved thoroughly in a boiling water bath. The medium was sterilized in an autoclave at 0.8 psi, 115^{o}C for 20 min. The liquefied medium was poured into sterilized petri dishes, allowed to harden and dried overnight at 37 ^{o}C. Agar was omitted when Z-CII liquid medium was prepared. That time, all reagents were mixed together in a volumetric flask and the pH was adjusted using 4% NaOH solution. Then, medium was sterilized in an autoclave at 0.8 psi, 115^{o}C for 20 min.

NB liquid medium (1000 mL)

Polypeptone	10.0 g
Beef extract	5.0 g
NaCl	5.0 g
Distilled water	1000 mL
pH	7.2

b) Preparation of NB liquid medium:

The reagents were mixed together in a volumetric flask and the pH was adjusted using 4% NaOH solution. Then, medium was sterilized in an autoclave at 0.8 psi, 115^{o}C for 20 min.

ASW (Artificial Sea Water- Herbst's formula)

	ASW	2 ASW
NaCl	30.0 g	600.0 g
KCL	0.7 g	14.0 g
$MgCl_2 \cdot 6H_2O$	10.8 g	216.0 g
$MgSO_4 \cdot 7H_2O$	5.4 g	108.0 g
$CaCl_2 \cdot 2H_2O$	1.0 g	20.0 g
Distilled water	1.0 L	10.0 L

Dilution solution (3/4 ASW)

2 ASW	375 mL
Distilled water	625 mL

c) Preparation of 2ASW and dilution solution (3/4 ASW)

All reagents were mixed and thoroughly dissolved.

3. Preparation of diatom double-layer agar plate

Diatom double-layer agar plates, described in the previous papers (Sakata *et al*., 1995, 1996 and 2000) were used for labyrinthulids isolation in this study. Diatom,

Chaetoceros ceratosporum C-16 was used in the double-layer agar medium. Diatom C-16 was stocked at 10 mL of ESS medium in growth chamber (SANYO) at 23^{o}C under 5000 lux light per 12L: 12D (12 hr light and 12 hr dark) cycle.

a) Culture preparation of *C. ceratosporum* C-16:

ES liquid medium (without ESP) 300 mL was taken in a 500 mL volumetric flask and 12 mL of ESP solution was taken in a test tube. After sterilized in an autoclave at 0.8 psi, 115^{o}C for 20 min, ESP was mixed with ES medium and made the medium cool down. Then 10 mL of *C. ceratosporum* (stocked) was poured into the flask and incubated at 23^{o}C of growth chamber (SANYO) in 5000 lux light per12L:12D cycle with aeration for 10 days.

b) Preparation of ES agar plates:

ES medium (without ESP) was taken in a beaker and 1.5% agar was added and dissolved thoroughly in a boiling water bath. Required amount of ESP was taken in a test tube. After sterilized in autoclave, both were mixed and made in water bath at 55^{o}C. Then, the medium was poured into sterilized petri dishes, allowed to harden and dried overnight at 37^{o}C.

c) Preparation of ES soft agar:

ES medium (without ESP) was taken in a beaker and 0.8% agar was added and dissolved thoroughly in a boiling water bath and was poured into stock tubes at amount of 2 mL each. Required amount of ESP was taken in a test tube. After sterilized, 80 µL of ESP was poured into each 2 mL stock tube of ES medium by micropipette.

d) Double layer agar plates preparation:

Cultured *C. ceratosporum* C-16 (from a) was collected in a beaker after centrifugation (3,000 x *g*, 10 min) and diluted by ES liquid medium to make 10 times

dilution of the cultured stock (300 mL). One mL of diluted *C. ceratosporum* was poured in 2 mL of ES (Provasoli's enrichment seawater medium) soft agar tube (which was melted by boil water and kept in 55^{o}C water bath) and mixed gently, then poured into ES agar plate and spread over the surface. After solidified it was kept in growth chamber (SANYO) under 5000 lux light per12L:12D cycle. Plate was sealed by Para film for long time stock.

4. Collection of samples and isolation of *Labyrinthula* strains

Sea water samples (0.1 mL) were mixed with soft agar medium containing ES medium, living diatom cells (*C. ceratosporum*) and 0.5% agar, and overplayed on basal agar medium (20 mL) containing ES medium and 1.5% agar in a petri dish. Plant and algae fragments (like *Ulva* sp., *Sargassum* sp., mangrove leaves and brown algae) were placed on the double layer agar plates prepared as the same method. The double layer agar plates were incubated at 23^{o}C under fluorescence light (5,000 lx, 12 h light and 12 h dark cycle). The plates were examined at 24 h intervals for the occurrence of labyrinthulid cells in the plaque area of diatom cell lysis (Figure II-1), which were then transferred aseptically to the fresh diatom agar plates. All labyrinthulid isolates were maintained in the double-layer agar plates with growing diatom cells in a incubator with fluorescence illumination at 23^{o}C.

Axenic culture of labyrinthulids on various media

Five mL of basal ES agar, which was composed of ES medium and 1.5% agar, was put in a 100 mL flask and autoclaved. Four types of agar media were used in the upper layer of the flask culture. Seven mL of 2% agar media including NSW, NSW with

bacterial dead cells and extract (NSB), NSW with egg yolk emulsion (NSEY), or NSW with bacterial dead cells and extract plus egg yolk emulsion (NSBEY) was overlaid on the basal layer. The cells of *Vibrio parahaemolyticus* VPHK-46 strain grown in 200 mL of marine broth Z-CII for 3 days were suspended in 40 mL of NSW and boiled for 10 min to prepare the bacterial dead cells and extract (NSB). Egg yolk removed from a whole egg aseptically was mixed with equal volume of sterilized NSW. All medium components except egg yolk emulsion were dispensed in small vials and sterilized by autoclaving.

Agar blocks containing labyrinthulid cells from the diatom double-layer agar plates were placed on the center of the double-layer agar in a flask and were incubated at 25^{o}C for 3 days. And then 20 mL of sterilized NSW and 5 mL of antibiotic solution (penicillin G, 2.5 g/L and streptomycin, 2.5 g/L, Wako, Japan) were added into the flask and the incubation continued up to 8 days. Labyrinthulid cells were collected from the overlaid NSW of the triple layer medium in a flask and the cell number in the NSW suspensions was directly counted on a Thoma hemocytometer under a light microscope at the 8th day of incubation. Aliquot (0.1 mL) of the NSW suspensions was spread on Z-CII agar medium containing 5 g peptone and 1 g yeast extract per 1000 mL artificial sea water to check bacterial contamination.

Phylogenetic analysis of labyrinthulid isolates based on 18S rDNA sequences

Total DNA of the labyrinthulid isolates was extracted according to Sakata *et al*. (2000). Polymerase chain reaction (PCR) amplification of 18S rDNA was carried out with a set of primers EuSSUF-1, 5'-AACCTGGTTGATYCTGCCAG-3', and EuSSUR-1, 5'-TGATCCTTCYGCAGGTTCACCTAC-3' (Sakata *et al*., 2000).

Thermal cycling was programmed as follows: 1 min at 95˚C for initial denaturation, followed by 30 cycles of 15 s at 95˚C, 30 s at 60˚C, and 1.5 min at 72˚C, with a final extension step for 7 min at 72˚C. The PCR products were subjected to sequencing reaction with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) and analyzed with the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, CA, USA). The nucleotide sequences were aligned with 18S rDNA sequences of the Order Labyrinthulida species and the isolates, and bootstrap resampling (1000 replicates) (Felsenstein, 1985), genetic distance calculation by Kimura's 2-parameter method (Kimura, 1980) and phylogenetic tree construction by the neighbor-joining method were carried out with a multiple sequence alignment program Clustal W 1.83 (Thompson *et al*., 1994). The phylogenetic tree was rooted with 18S rDNA sequences of *Giraudyospsis stellifera* (Chrysomerophyceae), *Oikomonas* sp. SA-2.1 (Oikomonadaxeae) and *Chromulina nebulosa* (Chrysophyceae) sequences as an outgroup.

Bacteriolysis of dead cells on double layer agar plates

Gram-positive bacteria including *Micrococcus* sp., *Corynebacterium* sp. and *Bacillus* sp., which were isolated from soil, were cultured in nutrient broth (NB) medium. Gram-negative bacteria including *Vibrio parahaemolyticus* VPHK-46, *Vibrio* sp. 3S1-13 and *Pseudoalteromonas* sp. A1-J25a, all of which were isolated from sea water of Kagoshima Bay, and *V. harveyi* (ATCC 14126) and *V. alginolyticus* (ATCC 17749), were cultured in Z-CII liquid medium as described by Phyu Phyu Than *et al*. (2004). After 3 days incubation at 30˚C, cells of each bacterium were collected by centrifugation and suspended in 3/4 strength of artificial seawater (ASW, Herbst's

formula composing of NaCl 30.0 g, KCl 7.0 g, $MgCl_2 \cdot 6H_2O$ 10.8 g, $MgSO_4 \cdot 7H_2O$ 5.4 g and $CaCl_2 \cdot 2H_2O$ 1.0 g per L). After 3 mL of the cell suspension with 0.8% agar was heat treated by autoclaving, it was overlaid on ES agar layer in a petri dish. The agar blocks containing labyrinthulid cells (00-Bat-05, 01-Jn-3a and 01-Jy-1b) were inoculated on the center of the bacterial dead cell plates and incubated in 25°C for 3 days. Bacteriolysis by labyrinthulid isolates was determined by the appearance of lytic zone around the agar blocks containing labyrinthulid cells on the plates.

Bacteriolysis of dead cells in liquid culture

Vibrio parahaemolyticus VPHK-46 cells grown in 400 mL of Z-CII medium for 3 days were collected by centrifugation (5,000 x *g*, 15 min) and washed with 40 mL of diluted ASW (3/4 strength). The bacterial cells obtained were suspended in 40 mL of diluted ASW and boiled for 10 min and centrifuged. Finally, the dead cell precipitate, after heat treatment, was suspended in sterilized ASW (3/4 strength) to adjust turbidity of the dead cell suspension at around 0.5 at 540 nm.

As shown in Figure II-2, 8 mL of the bacterial dead cell suspension, 2 mL of labyrinthulid cell suspension and 1 mL of antibiotics solution of penicillin G (2.5 g/L) and streptomycin (2.5 g/L) were added into an L-shape tube in order to prevent bacterial contamination. For the control (without *Labyrinthula* cells), 10 mL of the bacterial dead cell suspension and 1 mL of antibiotics solution of penicillin G (2.5 g/L) and streptomycin (2.5 g/L) were added into an L-shape tube. A duplicate set of test tubes was prepared for each test mixture and incubated at 25°C under rotary shaking. Turbidity at 540 nm was measured in every 2 h interval for 8 h and finally at 24 h of incubation. Bacteriolysis by strain 00–Bat-05 in L-shape tubes containing *V.*

parahaemolyticus dead cells was examined at various temperatures from 15 to 40˚C for 8 h. Data was expressed as lytic activity index calculated by the formula; lytic activity index = (initial absorbance – absorbance at 4 or 8 h with the test strain)/(initial absorbance – absorbance at 4 or 8 h without the test strain)

Effect of respiratory inhibitors on bacteriolysis by strain 00-Bat-05

Four types of inhibitors, sodium azide, sodium cyanide, ouabain and 2, 4-dinitrophenol, were used in this test and applied with high (1 mM) and low (0.1 mM) concentrations in the test suspension i.e. with *Labyrinthula*. Bacterial dead cell suspensions of *Vibrio parahaemolyticus* VPHK-46 and *Labyrinthula* (00-Bat-05 strain) suspension (live cell) were prepared in L-tubes for incubation as mentioned before and after that each chemical inhibitor was poured into test suspension separately. For control of dead cell suspension, only 10 mL of dead cell solution and 1 mL of antibiotic solution were added and another control was used for *Labyrinthula* suspension (without inhibitor). The initial turbidity of the mixture was adjusted around 0.5 at 540 nm. Then the mixture was incubated at 25°C under rotation and the absorbance at 540 nm was measured in every 2 h interval for 8 h and finally measured at 24 h.

To measure the effect of inhibitor on *Labyrinthula* cells during bacteriolysis, another set of experiment was set up as above protocol with one representative inhibitor, sodium azide with 1 mM concentration. After 24 h, *Labyrinthula* cells with and without of sodium azide were collected by centrifugation and reused with new dead cells of *V. parahaemolyticus* to check the bacteriolysis capability of *Labyrinthula* cells strain 00-Bat-05.

Hydrolysis test

Four isolated strains, 01-Jn-3a and 01-Jy-1b from Japan and 00-Bat-03 and 00-Bat-05 from the Philippines, were subjected for the hydrolysis test. Test for hydrolysis of starch, casein, chitin and tri-n-butyrin were done on ESS double-layer agar plates. ESS agar medium (1.5% agar) was used in the basal layer of the plate. The mixture of dead cells of VPHK-46 strain, 0.5% of agar and each chemical was used on the upper layer. Starch 0.5%, casein (skim milk) 2.0%, chitin 1.0% and tri-n-butyrin 2.0% were used separately in the double layer agar plates. One mL of antibiotic solution (solution of 0.25% Penicillin and Streptomycin) was added in the medium before pouring on the plate. *Labyrinthula* strains were inoculated on two spots of the plate and incubated for seven days at 25^{o}C. A positive result is determined by the appearance of a lytic zone on the plate, signifying casein, chitin and tri-n-butyrin hydrolysis.

Enzyme test by API-ZYM kit

Four isolates, 01-Jn-3a and 01-Jy-1b strains from Japan and 00-Bat-03 and 00–Bat-05 strains from Philippine, were selected to determine the enzymatic reaction of labyrinthulids. In this test, API-ZYM test kit (BioMérieux, France) was used. The 7 days cultures of each *Labyrinthula* strains from ESS liquid media were centrifuged (5,000 x *g*, 15 min.). *Labyrinthula* cells were collected and suspended in 2 mL of ¾ ASW (turbidity 4 – 5 of McFarland standard, BioMérieux, France) and then 65 µL of each strain was dispensed into each of the 20 API-ZYM strip wells. The strips were incubated at 25^{o}C for 5 h in the opposite API-ZYM chamber humidified with distilled water. After this incubation period a drop of each of ZYM A (BioMérieux, France) and ZYM B reagents (BioMérieux, France) were added to each of the twenty wells. The

color reaction was read after one hour incubation at 25°C, according to the API-ZYM kit's reading color-scale (Table II-3). A symbol ranging from – to +++ was assigned, corresponding to the developed color: - (negative) corresponds to the negative reaction, +++ to the maximum intensity reaction and symbols ±, + or ++ correspond to the results of intermediate reactions.

Pigment extraction

Orange-pigmented labyrinthulid cells grown at 25˚C for 3 days under rotary shaking in an L-shape tube added with 10 mL of the bacterial dead cell suspension described above were collected by centrifugation at 5,000 x *g* for 15 min. The labyrinthulid cells were suspended in 30 mL of ethanol and then kept in refrigerator for 3 h. After filtration with paper filter (No. 5A, Advantec, Japan), 30 mL of filtrated ethanol solution, 20 mL of *n*-hexane and 40 mL of acetone were mixed vigorously in a separating funnel and the mixture was washed 5 times with 90 mL of distilled water and then the upper *n*-hexane layer of the mixture was collected. Then pigment component in *n*-hexane was concentrated with evaporator or with N_2 flashing and dissolved in acetone. Finally visible absorption spectrum of the pigment dissolved in acetone was measured by a spectrophotometer (MPS-2000, Shimadzu Corp., Japan)

Results

Growth response of labyrinthulid isolates on various agar media

A total of 13 strains of labyrinthulids were isolated from different marine environments of Japan and a mangrove area in the Philippines as shown in Table 1.

Among 11 strains collected from Kagoshima Bay, two were originated from coastal seawater, three from green algae (*Ulva* spp.), two from brown algae (*Sargassum* spp.) and four from submerged mangrove leaves (*Kandelia* sp.). The other two strains were isolated from submerged mangrove leaves in Batan Bay, the Philippines.

Plaque zones were observed surrounding algae or leaf fragments on the double layer agar plates containing living diatom cells during 6 to 12 days of incubation. It was found that the large and medium-sized plaques in the diatom lawn composed of growing diatom cells were mainly produced by labyrinthulids, amoebae or filamentous bacteria. Labyrinthulid plaques were distinguished from other types of plaque by observing labyrinthulid cells under a microscope. On the other hand, the small and tiny plaques were formed mostly by rod-shaped bacteria.

With the aim of collecting large amount of axenic cells of labyrinthulid isolates, agar blocks containing labyrinthulid cells from the diatom double layer agar plates were inoculated to the triple layer medium of a flask. Cell counts of 01-Jn-3a and 00-Bat-05 strains after 8 days incubation were found to be relatively higher in NSBEY medium (2.7×10^6 and 5.3×10^6 cells/mL, respectively) than that in NSEY medium (1.4×10^5 and 1.0×10^6 cells/mL, respectively) as shown in Figure II-3. The Philippine isolate, 00-Bat-05 exhibited a better growth performance than the Japan isolates 01-Jn-3a in the media tested. In this experiment, any bacterial living cells were not observed in the NSW liquid layers of all test culture under a microscope and colony formation of bacteria was not found on Z-CII agar plates, on which aliquot of the NSW liquid layers was spread.

Taxonomic position of labyrinthulid isolates

In order to clarify the molecular phylogenetic positions of the representative isolates 01-Jy-1b, 00-Bat-03, and 00-Bat-05 (Accession numbers: AB290457, AB290458 and AB290459, respectively), a phylogenetic tree was constructed based on the 18S rDNA sequences as shown in Figure II-4. Loci of 1360 nucleotides that were unambiguously aligned were used for calculation. All the isolates belonged to the labyrinthulid phylogenetic group (Honda *et al*., 1999). The isolates 00-Bat-03 and 00-Bat-05 derived from the same mangrove leaf in the Philippines had almost the same sequences and align separately with the isolate 01-Jy-1b isolated from a mangrove leaf in Kagoshima Bay and *Labyrinthula* sp. AN-1565, reflecting the difference of these isolates' origins.

Bacteriolysis on double-layer agar plates

As shown in Figure II-5, the lytic zones by labyrinthulid isolates on the double-layer agar plates containing the dead cells of Gram-negative bacteria were observed under the translucent spread colony of labyrinthulid growing cells. Lytic zones for Gram-negative bacteria by 00-Bat-05 strain ranged from 34.8 to 56.3 mm in diameter depending on bacterial species, while those formed by strain 01-Jn-3a and 01-Jy-1b ranged from 45.8 to 58.0 mm and 6.8 to 9.6 mm, respectively (Table II-2). Diameters of lytic zones were always found to be smaller than that of spread colony of labyrinthulid isolates. Strain 00-Bat-05 formed colony consisting of relatively low density of growing cells on agar plates with the dead cells of Gram-positive bacteria but was unable to produce a lytic zone, suggesting that it can utilize some nutrient including in the bacterial cell extract. Both strains of 01-Jn-3a and 01-Jy-1b could hardly grow on and lyse the dead cells of Gram-positive bacteria.

In bacteriolytic activity tests for labyrinthulid isolates on the double-layer agar plates containing the dead cells of Gram-positive or Gram-negative bacteria, it was found that labyrinthulid isolates such as 00-Bat-05, 01-Jn-3a and 01-Jy-1b could lyse only the dead cells of Gram-negative bacteria including *V. parahaemolyticus* VPHK-46, *V. harveyi* (ATCC 14126), *V. alginolyticus* (ATCC 17749), *Vibrio* sp. 3S1-13 and *Pseudoalteromonas* sp. A1-J25a, but did not lyse those of Gram-positive bacteria including *Micrococcus* sp., *Corynebacterium* sp. and *Bacillus* sp. during a incubation period.

Bacteriolysis in bacterial cell suspensions

Comparison of bacteriolysis performances of strain 00-Bat-05 for Gram-positive and Gram-negative bacteria in the bacterial dead cell suspensions revealed that the labyrinthulid isolate could lyse only Gram-negative bacteria as shown in Figure II-6. Turbidity of the dead cell suspensions of *V. parahaemolyticus* decreased from 0.47 to 0.18 (63% decrease) at 8 h and to 0 (100% decrease) at 24 h incubation, while that of *Micrococcus* sp. suspension decrease from 0.47 to 0.39 (17% decrease) at 8 h and to 0.38 (19%) at 24 h.

In order to assess the effect of temperature on bacteriolysis by strain 00-Bat-05, bacteriolytic activity was determined at different temperatures (15 to 40^{o}C). Lytic activity index was found highest at 31^{o}C and lowest at 40^{o}C as illustrated in Figure II-7. For both 4 and 8 h incubations, lytic activity index increased proportionally with increasing temperature from 15 to 31^{o}C. The optimum temperature range for bacteriolysis by strain 00-Bat-05 was shown to be from 25 to 31^{o}C. Apparently lytic activity index began to decrease above 31^{o}C.

Effect of respiratory inhibitors on bacteriolysis by strain 00-Bat-05

Different types of respiratory inhibitors were used in the test of bacteriolysis. Sodium azide and ouabain are known as classical ATPase inhibitors. Sodium cyanide is known as respiratory inhibitor and 2, 4-dinitrophenol is used as pesticide. Dinitrophenol was found as the strongest inhibitor at both high (1 mM) and low (0.1 mM) dose in bacteriolysis by *Labyrinthula* (Figure II-8). Sodium azide and sodium cyanide were also showed the same inhibition at high dose (1 mM) but lower inhibitions were found in low dose (0.1 mM). The lowest inhibition was found in ouabain i.e. it did not effect the lytic activity of *Labyrinthula*.

To check the effect of sodium azide on *Labyrinthula* cells during bacteriolysis, it was found that *Labyrinthula* cells were inactive or bacteriolysis did not observe when sodium azide was present (Figure II-9.A). But after 24 h, when *Labyrinthula* cells were recollected from the sodium azide condition and same *Labyrinthula* cells were incubated with bacterial dead cell suspension, it became active again (Figure II-9.B). It means that sodium azide acts as a biostatic on *Labyrinthula* cells by inhibiting respiration.

Hydrolytic and API-ZYM test

In hydrolysis test, four representative strains (strain 00-Bat-03, 00-Bat-05, 01-Jn-3a and 01-Jy-1b) decomposed starch and two strains 00-Bat-05 and 01-Jn-3a decomposed casein. But none of them (4 strains) could hydrolyze chitin and try-n-butyrin as shown in Table II-4.

API-ZYM test kit is used for semi-quantitation of enzymatic activities in various microorganisms. In enzymatic reaction test, investigated strains of *Labyrinthula* were

characterized by activity of 10 hydrolytic enzymes (Table II-4). None of the strains exhibited activity of trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The higher activity was shown in alkaline phosphatase in all strains. Other two enzymes, leucine arylamidase and valine arylamidase were also found in higher level in strain 00-Bat-03, 00-Bat-05 and 01-Jy-1b, while valine arylamidase was found in medium level and leucine arylamidase found in low level in strain 01-Jn-3a. Three enzymes such as esterase lipase, acid phosphatase and naphthol-AS-BI-phosphohydrolase were found in medium level in strain 00-Bat-03, 00-Bat-05, 01-Jy-1b and 01-Jn-3a (only in esterase lipase), while acid phosphatase and naphthol-AS-BI-phosphohydrolase were found in very low level in strain 01-Jn-3a. Four enzymes such as esterase, lipase, cystine arylamidase and β-glucosidase were found in low level in strain 00-Bat-03, 00-Bat-05 and 01-Jy-1b, while three enzymes (esterase, lipase, and β-glucosidase) were found in very low level and negative activity of cystine arylamidase was shown in strain 01-Jn-3a . However, the study of enzymatic profile of labyrinthulids has not yet been reported.

Pigment and zoospore production in L-shape tube culture

Orange pigment production occurred in strain 00-Bat-05 cells attaching to the inner surface of an L-shape tube during 3 days incubation with the bacterial dead cells of *V. parahaemolyticus.* The pigment was extracted from the cells using ethanol and showed a specific absorption spectrum with maximum absorbance at 455 nm in acetone, indicating that strain 00-Bat-05 accumulates carotenoid pigment in liquid culture with bacterial dead cells during stationary growth phase (Figure II-10).

After orange pigment production completed in vegetative cell aggregates attaching to the glass wall of a test tube, rapid movement of zoospore (meiospore) of strain 00-Bat-05 with biflagella was observed with a light microscope. Cells of zoospore are smaller spherical shape (2-3 μm) compared with spindle shape of vegetative cells (3-5 x 12-16 μm) as shown in Figure II-11.

Discussion

Labyrinthula spp. are known to be widely distributed in the marine environments and are commonly isolated from sea grasses, submerged mangrove leaves and macroalgae with fewer isolates from seawater column. In this study 13 strains of labyrinthulids were isolated from different marine environments of Japan and a mangrove area in the Philippines. Plaque zones appeared on the diatom double layer agar plates after 5-7 days incubation at 23°C included clumps of spindle-shaped cells of labyrinthulids as described by Sakata and Iwamoto (1995).

To obtain the labyrinthulid cells more effectively, various media compositions were examined for labyrinthulid growth media. Relatively higher cell yield was obtained for the NSBEY medium composed of natural seawater, dead cells and extract of Gram-negative bacterium such as *V. parahaemolyticus*, and egg yolk emulsion. Muehlstein *et al*. (1988) proposed serum seawater broth (SSB) containing 1% horse serum for axenic cultures of *Labyrinthula* spp. However, both of bacterial dead cells and egg yolk used in this study are easily and cheaply available at any time. These results suggested that Gram-negative bacteria cells, egg yolk or horse serum contain stimulating or essential components for the growth of labyrinthulid isolates.

As their unusual nature, *Labyrinthula* spp. were reported to be capable of decomposing various microbial living cells including yeast, fungal hyphae, microalgae and bacteria in laboratory condition and found to prefer Gram-negative bacteria as food (Yokochi *et al*., 2002; Perkins and Amon, 1969; Perkins, 1973). In hydrolysis test, it had been found that labyrinthulid isolates could hydrolyse only macromolecular compounds like starch but could not complex compounds like chitin and try-n-butyrin. Fujisawa (1998) also found the same phenomenon in *Labyrinthula* strain L95-1. L95-1 had shown positive results in starch and casein and negative results in chitin and try-n-butyrin. In bacteriolysis tests for labyrinthulid isolates on double-layer agar plates containing bacterial dead cells they decomposed the dead cells of Gram-negative bacteria, and not of Gram-positive bacteria. These results suggest that *Labyrinthula* spp. could not lyse Gram-positive bacteria cells with the comparatively thick peptidoglycan layer as the major component of their cell walls. On the other hand, the cell wall of Gram-negative bacteria consists of protein- and lipid-rich components such as lipoprotein and lipopolysaccharides (LPS) and these components could be susceptible to attack by *Labyrinthula* enzymes. Klie and Mach (1968) examined the growth of *L. coenocystis* on bacteria and yeast species and reported that it could easily utilize all of the yeasts and Gram-negative bacteria, but were unable to degrade Gram-positive bacteria. Perkins and Amon (1969) reported that the lytic activity of *Labyrinthula* spp. depended on their ectoplasmic network when other microorganism cells were present as a food source.

During 3 days incubation of the strain 00-Bat-05 with the bacterial dead cells of *V. parahaemolyticus* as sole nutrient source, orange pigment was accumulated in the cell aggregates attaching to the inner wall surface of an L-shape tube. After the pigment

production completed in cell clumps of the strain 00-Bat-05, rapid movement of zoospore was recognized within cell clumps under a light microscope, suggesting that after labyrinthulid cells reach stationary phase of growth, pigment production proceeds and followed by zoospore formation. The labyrinthulid strains used in this study were never observed to produce zoospores on the double-layer agar plates containing living diatom cells. Perkins and Amon (1969) described that sporulation was initiated by aggregation of spindle cells to form sporangia, in which successive bipartition resulted in 8 biflagellated zoospores. However, the physiological relationship between the carotenoid pigmentation and cell differentiation of labyrinthulids remains to be established.

This study makes clear that labyrinthulids can grow axenically and produce zoospores during incubation with bacterial dead cells suspension, while on the double-layer agar containing living diatom cells they can grow vigorously but have been hardly observed zoospore formation during incubation. However, it has not elucidated which components or metabolites of bacterial dead cells suspension stimulate the growth, pigment production and zoospore formation.

Table II-1. Sample sources and labyrinthulid isolates from marine environments

Isolate	Sample	Sampling site	Date	Water temp. (°C)	Plaque No. / g or mL
01-Ap-2	Seawater	Shigetomi, Kagoshima	Apr. 10, 2001	21	6.0×10^2
01-Ap-3b	Green alga	Shigetomi, Kagoshima	Apr. 10, 2001	21	6.9×10^2
01-My-1b	Seawater	Prawn tank, Kagoshima	May 10, 2001	22	7.9×10^2
01-Jn-1a, 01-Jn-1b	Green alga	Takoyama, Kagoshima	Jun. 6, 2001	28	6.7×10^3
01-Jn-3a, 01-Jn-3b	Mangrove leaves	Nukumi , Kagoshima	Jun. 22, 2001	22	5.5×10^3
01-Jn-4a, 01-Jn-4b	Brown alga	Sesegushi, Kagoshima	Jun. 22, 2001	25	9.7×10^4
01-Jy-1a, 01-Jy-1b	Mangrove leaves	Sesegushi, Kagoshima	Jul. 10, 2001	24	1.5×10^2
00-Bat-03, 00-Bat-05	Mangrove leaves	Batan Bay, Philippines	Dec. 8, 2000	ND*	ND

*ND, not determined

Table II-2. Bacteriolytic activity of labyrinthulid isolates for the dead cells of Gram-positive and Gram-negative bacteria

Bacterial strains		Colony diameter and lytic zone of labyrinthulid isolates					
		00-Bat-05		01-Jn-3a		01-Jy-1b	
		CD[*1]	LZ[*2]	CD	LZ	CD	LZ
Gram-positive	*Micrococcus* sp.	47.4	N[*3]	N	N	N	N
	Corynebacterium sp.	56.7	N	8.6	N	N	N
	Bacillus sp.	60.3	N	N	N	N	N
Gram-negative	*Vibrio parahaemolyticus* VPHK-46	76.7	56.3	62.0	50.4	10.8	9.6
	Vibrio harveyi (ATCC 14126)	61.7	45.0	73.2	58.0	10.5	9.2
	Vibrio alginolyticus (ATCC 17749)	63.0	51.3	64.2	48.0	7.2	6.8
	Vibrio sp. 3S1-13	43.7	34.8	43.0	N	N	N
	Pseudoalteromonas sp. A1-J25a	79.5	47.7	75.9	45.8	4.6	N

[*1]CD, colony diameter (mm); [*2]LZ, lytic zone diameter (mm); [*3]N, no growth. Values indicate averages of 2 plates.

Table II-3. Interpretation of the enzymatic tests (API-ZYM kit)

No.	Enzyme assayed	Substrate	pH	Result: Positive	Result: Negative
1	Control			No color or color of the sample if it has an intense coloration	
2	Alkaline phosphatase	2-naphthyl phosphate	8.5	purple	No color or color of the control - if the strip has been exposed to an intense light source after addition of the reagents. Very pale yellow - if the strip has not been
3	Esterase (C4)	2-naphthyl butyrate	6.5	purple	
4	Esterase lipase (C8)	2-naphthyl caprylate	7.5	purple	
5	Lipase (C14)	2-naphthyl myristate	7.5	purple	
6	Leucine arylamidase	L-leucyl-2-naphthylamide	7.5	orange	
7	Valine arylamidase	L-valyl-2-naphthylamide	7.5	orange	
8	Cystine arylamidase	L-cystyl-2-naphthylamide	7.5	orange	
9	Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	8.5	orange	
10	Chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide	7.5	orange	
11	Acidic phosphatase	2-naphtyl phosphate	5.4	purple	
12	Naphthol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate	5.4	blue	
13	α-galactosidase	6-Br-2-naphtyl-α-D-galactopyranoside	5.4	purple	
14	β-galactosidase	2-naphthyl-β-D-galactopyranoside	5.4	purple	
15	β-glucuronidase	Naphthol-AS-BI-β-D glucuronide	5.4	blue	
16	α-glucosidase	2-naphthyl-α-D-glucopyranoside	5.4	purple	
17	β-glucosidase	6-Br-2-naphthyl-β-D-glucopyranoside	5.4	purple	
18	N-acetyl-β-glucosaminidase	1-naphthyl-N-acetyl-β-D-glucosaminide	5.4	brown	
19	α-mannosidase	6-Br-2-naphthyl-α-D-mannopyranoside	5.4	purple	
20	α-fucosidase	2-naphthyl-α-L-fucopyranoside	5.4	purple	

Table II-4. Hydrolysis and API-ZYM tests of *Labyrinthula* strains

	Labyrinthula strain			
	00-Bat-03	00-Bat-05	01-Jn-3a	01-Jy-1b
Hydrolysis test				
Starch	+	+	+	+
Casein	-	+	+	-
Chitin	-	-	-	-
Tri-n-butyrin	-	-	-	-
API-ZYM test				
Alkaline phosphatase	+++	+++	+++	+++
Esterase	+	+	±	+
Esterase lipase	++	++	++	++
Lipase	+	+	±	+
Leucine arylamidase	+++	+++	+	+++
Valine arylamidase	+++	+++	++	+++
Cystine arylamidase	+	+	-	+
Acidic phospatase	++	++	±	++
Napthol-AS-BI-phosphohydrolase	++	++	±	++
β-Glucosidase	+	+	±	+

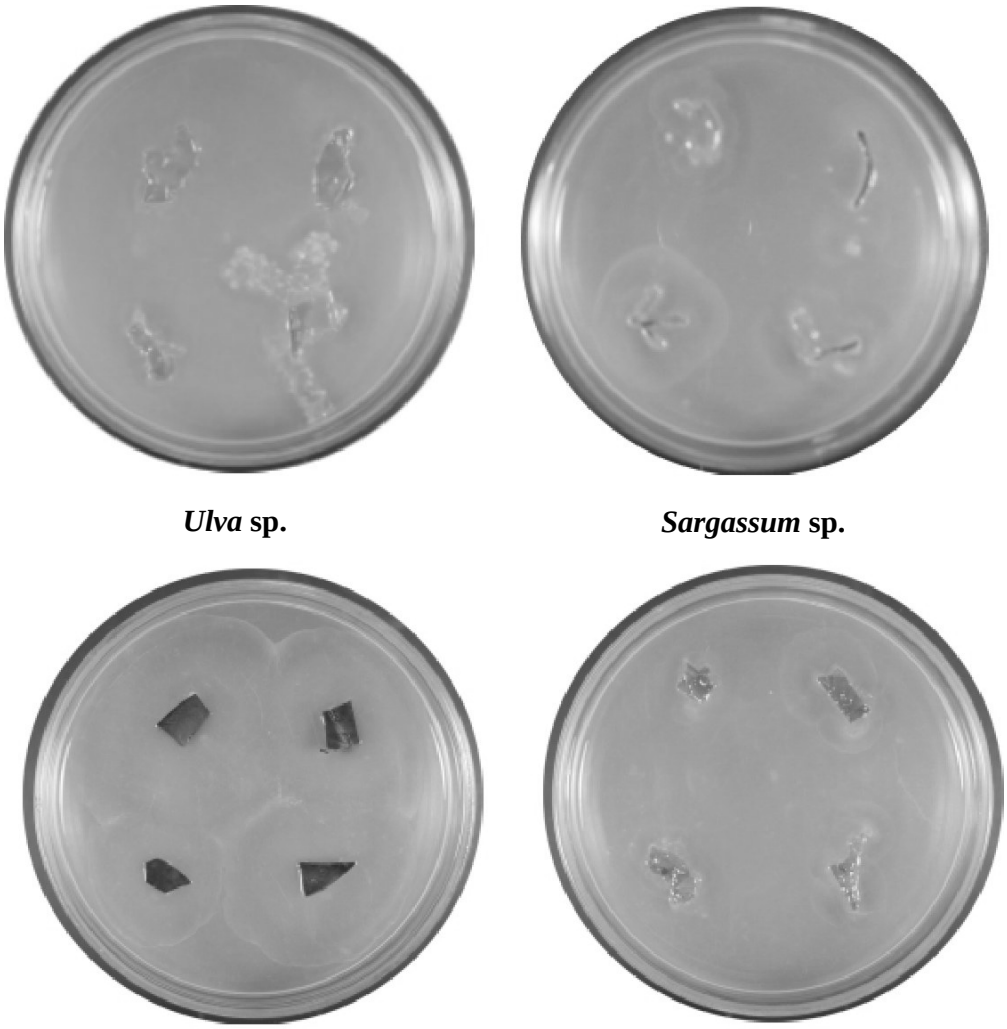


Figure II-1. Plaques of labyrinthulids from algae or plant samples.

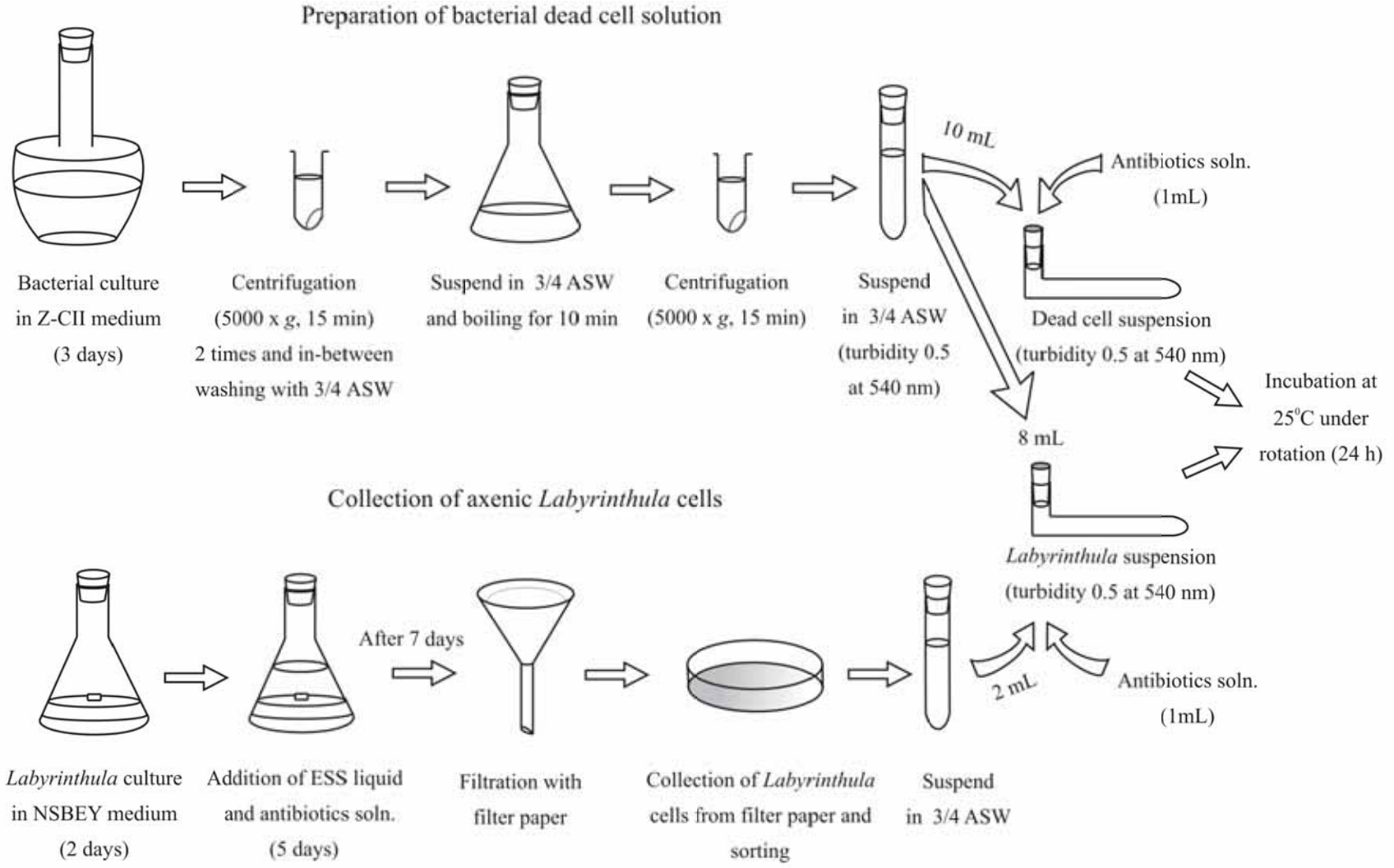


Figure II-2. Protocol of liquid culture of *Labyrinthula* cells for bacteriolysis.

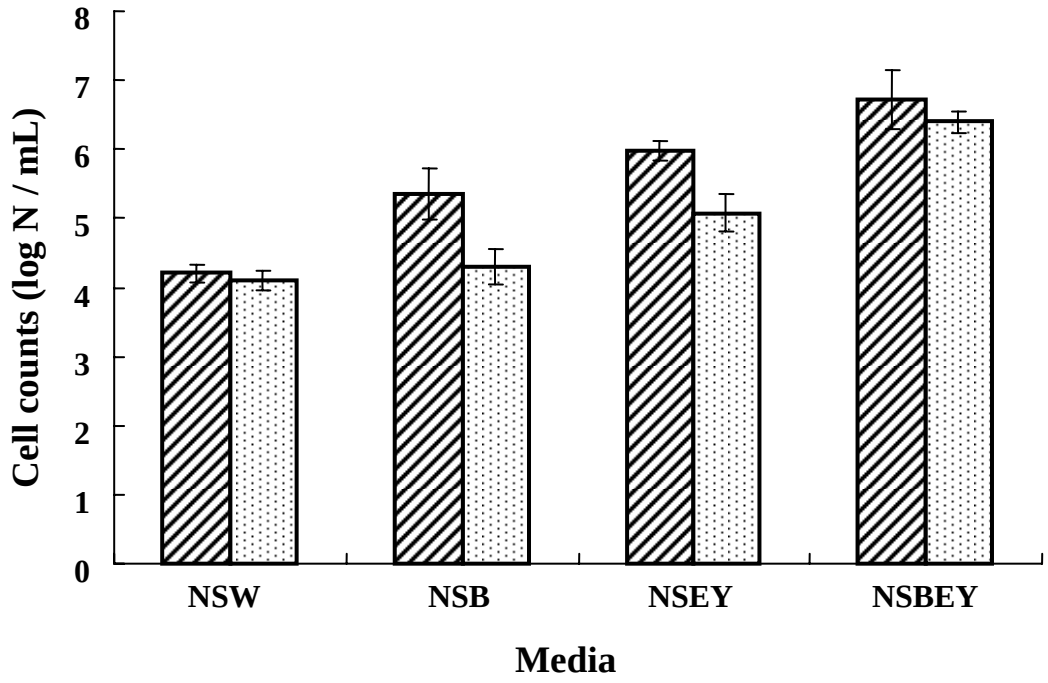


Figure II-3. Cell counts of the labyrinthulid isolates 00-Bat-05 and 01-Jn-3a in NSW suspensions after 8 days incubation in the triple layer media. Media: NSW, natural seawater; NSB, NSW with the bacterial dead cells and extract; NSEY, NSW with egg yolk emulsion; NSBEY, NSW with the bacterial dead cells and extract and egg yolk emulsion. Shaded columns, strain 00-Bat-05; dotted columns, strain 01-Jn-3a. Bars indicate standard deviation (SD) among 3 flasks.

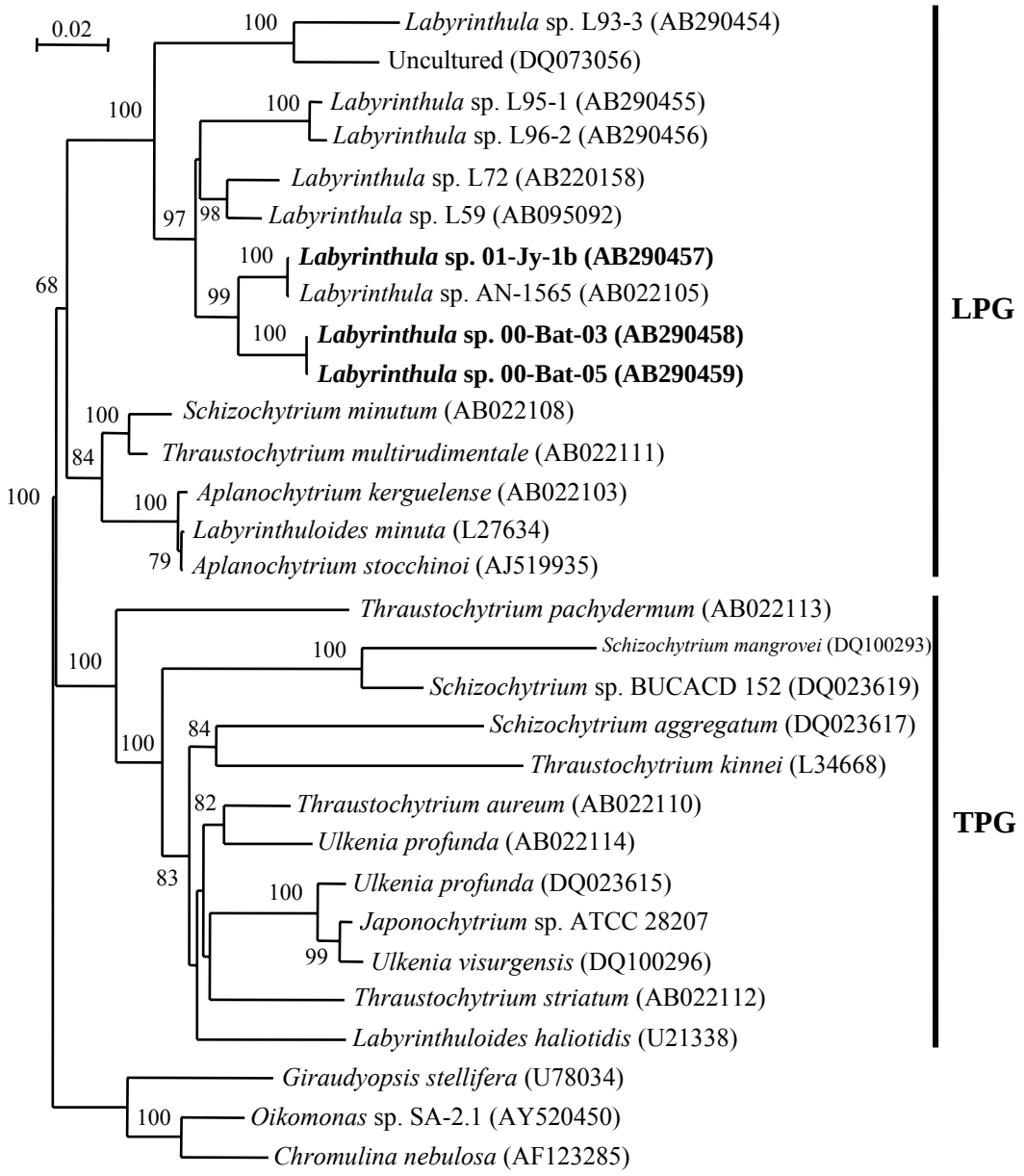


Figure II-4. Phylogenetic tree showing relationships among labyrinthulid isolates and Labyrinthulida representatives based on partial 18S rDNA nucleotide sequences. Bootstrap values more than 50% are shown at the nodes. Scale bar indicates the genetic distance (Knuc, substitution/site). Accession numbers of the nucleotide sequence database are shown in the parentheses. LPG, labyrinthulid phylogenetic group; TPG, thraustochytrid phylogenetic group (Honda, 1999).

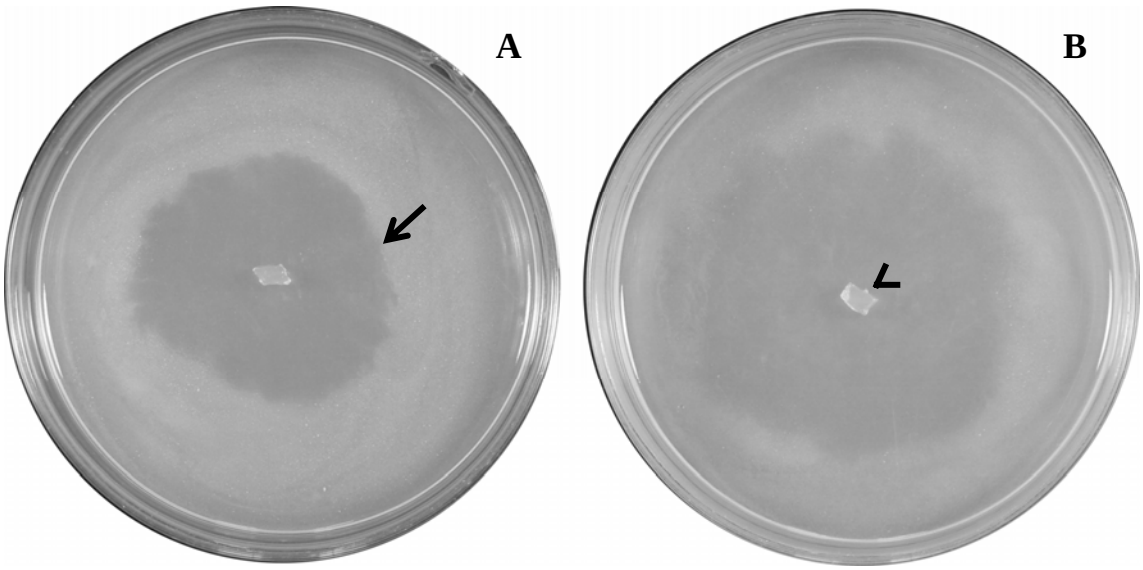


Figure II-5. Lytic zones by labyrinthulid isolates on the double-layer agar plates containing *V. parahaemolyticus* dead cells. A, strain 01-Jn-3a; B, strain 00-Bat-05. Arrow indicates a lytic zone and arrowhead indicates an agar block containing labyrinthulid cells for inoculation.

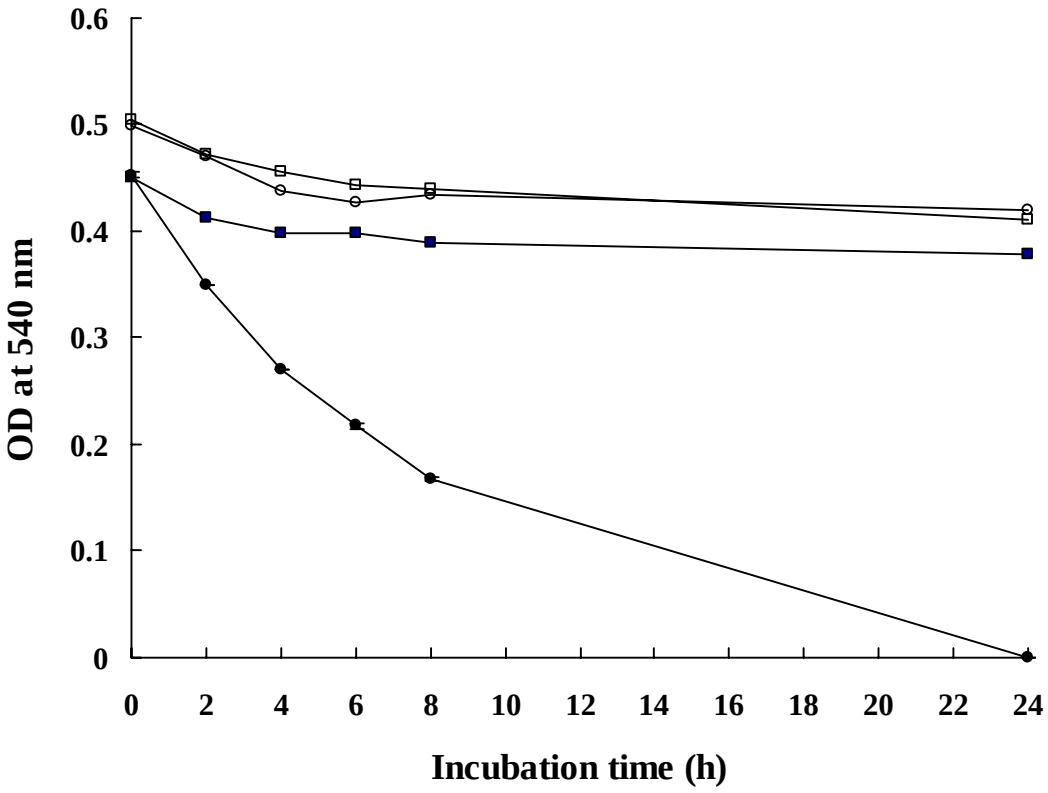


Figure II-6. Bacteriolysis by the labyrinthulid isolate 00-Bat-05 in L-shape tubes containing the dead cell suspensions of *Micrococcus* sp. or *V. parahaemolyticus*. □, dead cells of *Micrococcus* sp. without labyrinthulid cells; ■, dead cells of *Micrococcus* sp. with labyrinthulid cells; ○, dead cells of *V. parahaemolyticus* without labyrinthulid cells; ●, dead cells of *V. parahaemolyticus* with labyrinthulid cells. Values indicate averages for duplicate tubes.

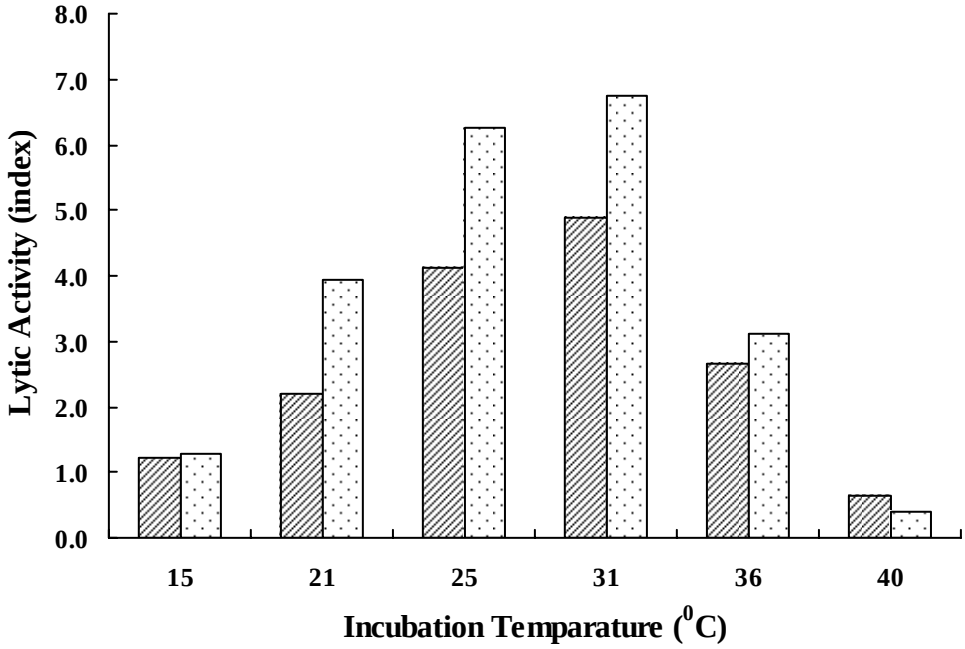


Figure II-7. Effect of incubation temperature on bacteriolysis by strain 00-Bat-05 in L-shape tubes containing *V. parahaemolyticus* dead cells. Shaded columns, 4 h incubation; dotted columns, 8 h incubation. Lytic activity index was calculated by the formula, Index = (initial absorbance – absorbance at 4 or 8 h with strain 00-Bat-05) / (initial absorbance – absorbance at 4 or 8 h without strain 00-Bat-05). Values indicate averages of duplicate samples.

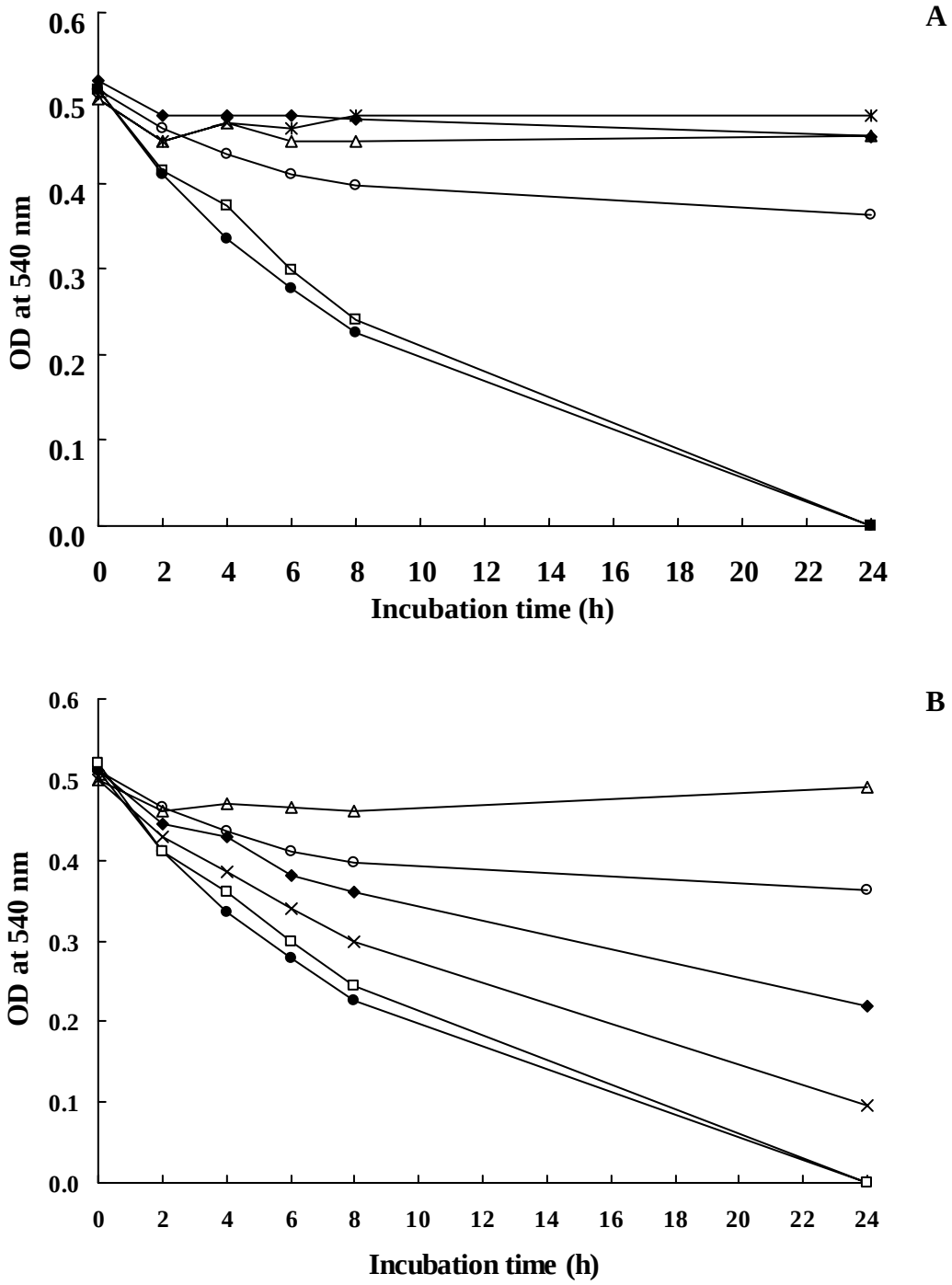


Figure II-8. Effect of respiratory inhibitors on bacteriolysis by strain 00-Bat-05. The concentration of the inhibitors in A, 1.0 mM and in B, 0.1 mM. ○, dead cells of *V. parahaemolyticus* (Vp) without labyrinthulid cells; ●, dead cells of Vp with labyrinthulid cells (Vp+laby); □, Vp+laby with ouabain; △, Vp+laby with dinitrophenol; ×, Vp+laby with sodium cyanide; ◆, Vp+laby with sodium azide. Values indicate averages for duplicate tubes.

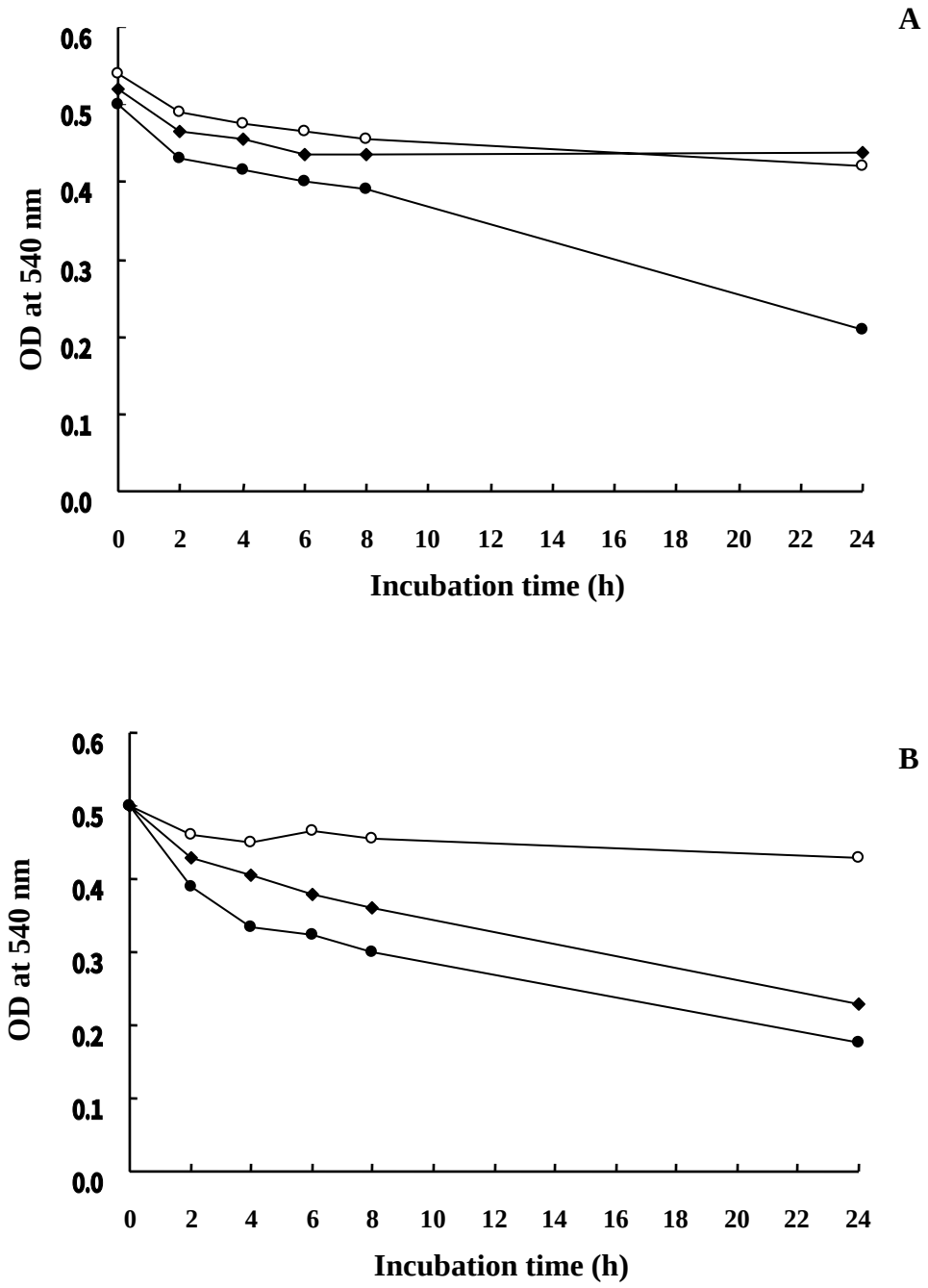


Figure II-9. Effect of sodium azide on bacteriolysis and strain 00-Bat-05 cells. The concentration of the inhibitor is 1.0 mM. In A, ○, dead cells of *V. parahaemolyticus* (Vp) without labyrinthulid cells; ●, dead cells of Vp with labyrinthulid cells (Vp+laby); ◆, Vp+laby with sodium azide. In B, Labyrinthula cells are used from A, under same condition without sodium azide (◆). Values indicate averages for duplicate tubes.

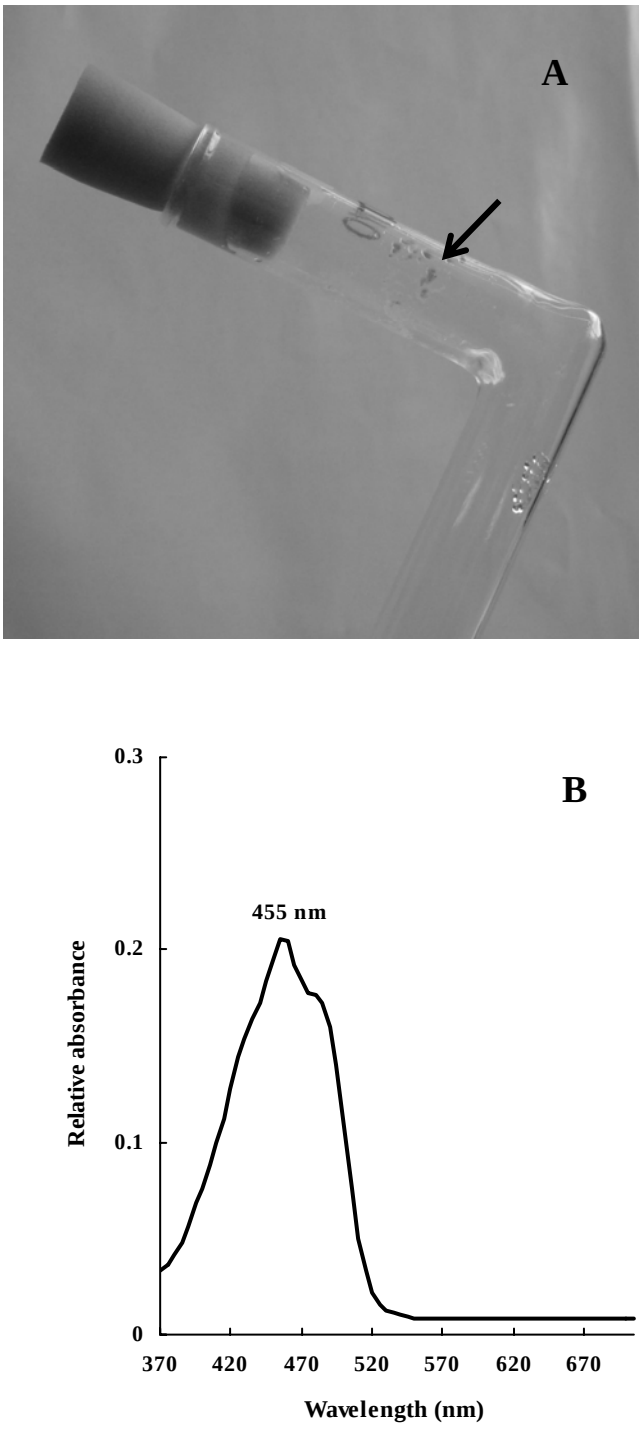


Figure II-10. Pigmentation of strain 00-Bat-05 grown in an L-shape tube containing *V. parahaemolyticus* dead cell suspension at 25^0C for 3 days. A, photograph of cell aggregates attaching on the inner surface of an L-shape tube (indicated with an arrow); B, absorption spectrum in acetone of pigment extracted from the cells.

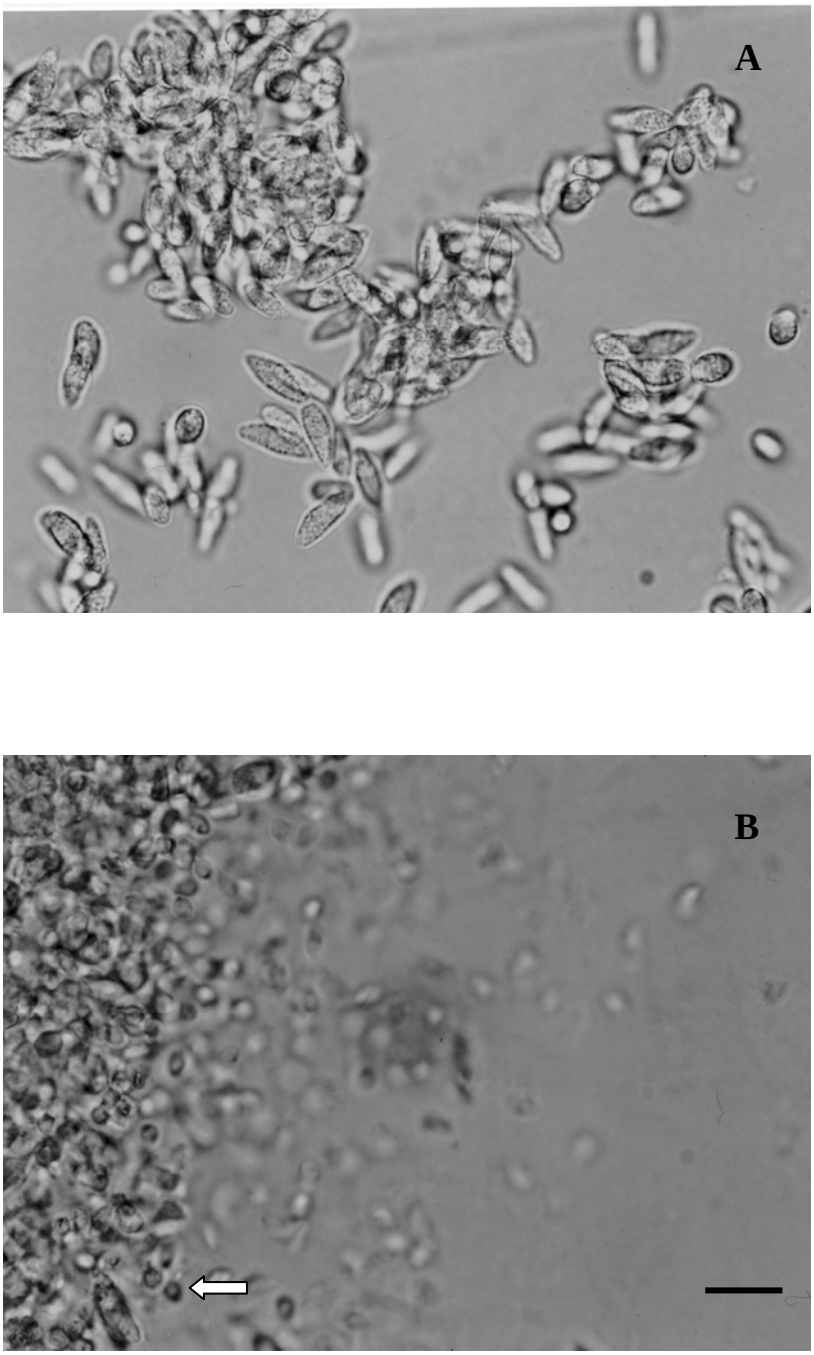


Figure II-11. Micrographs of vegetative cells and zoospores of strain 00-Bat-05 grown in an L-shape tube containing *V. parahaemolyticus* dead cell suspension. A, vegetative cells after 2 days incubation; B, zoospores after 4 days incubation (indicated with arrows). Bar indicates 10 μm.

Chapter III

Comparative Study of

Leucine Aminopeptidases from

Marine Labyrinthulid and Thraustochytrid Strains

Introduction

Labyrinthulids (Family Labyrinthulaceae) and thraustochytrids (Family Thraustochytriaceae) are the main two groups of the order Labyrinthulales and play the peculiar roles in marine food web systems (Porter, 1990; Cavalier-Smith, 1993; Honda *et al.*, 1999). Labyrinthulids are slime mold-like protists and distributed widely in estuarine and near-shore marine habitats throughout the world. In mangrove brackish water environments, *Labyrinthula* spp. live on mangrove leaves, marine vascular plants and benthic algae, and also parasitizes fungal hyphae, which are decomposing plant matter. *Labyrinthula* spp. play an important role in mangrove ecosystem by fixing plant nutrients in a form of hyphae or cells of the organisms and converting them to a nutritious food for plant consuming animals (Nakagiri, 2001). The ectoplasmic networks of *Labyrinthula* spp. are capable of decomposing many different microbes including bacteria, yeast, diatoms, and fungal hyphae (Porter, 1990). Thraustochytrids are a group of eukaryotic marine microorganisms, which can play dual roles in nature as bacterial feeders when in an ameboid form, and organic material degraders in their thallus form (Raghukumar, 1992). In mangrove environments, these marine protists are found to be associated with decaying mangrove leaves and decompose mangrove leaves by their degradative enzymes and also to be capable of breaking down several complex organic substrates (Bremer and Talbot, 1995; Raghukumar *et al.*, 1995, 2001).

In addition, these organisms also deserve more attention in aquaculture because they accumulate high concentrations of polyunsaturated fatty acids such as docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA) which are essential nutrients for many fish and crustaceans (Singh and Ward, 1997; Nakahara et al., 1996; Sakata *et al*., 2000; Miller *et al*., 2007). Thraustochytrids are used practically as a feed for zooplankton such

as rotifers and *Artemia* (Hayashi, 2002) because of improving the nutritional value of the food animals.

In natural ecosystems, the recycling of nutrients, especially protein degradation is important to maintain all living things and aminopeptidases are found widely distributed among both prokaryotic and eukaryotic organisms, which catalyze the release of amino acid residues from the N-terminal end of proteins (Gonzales and Robert-Baudouy, 1996). Leucine aminopeptidase (LAP, EC 3.4.11.1) activity in marine environments indicated an ecological role by bacteria in aquatic biogeochemical cycles (Caruso and Zaccone, 2000). LAP was partially purified from pig intestinal mucosa at first and found to hydrolyze a wide variety of peptides. The LAP activities from swine kidney and *Aspergillus sojae* was determined to be activated by divalent cations like Zn^{2+} and Co^{2+} (Himmelhoch, 1969; Chien *et al*., 2002).

The aim of this chapter of the study was to characterize and compare the leucine aminopeptidase (LAP) from marine labyrinthulid and thraustochytrid isolates in order to assess nutritional process and degradation activity of these organisms in marine environments.

Materials and Methods

Labyrinthulid and thraustochytrid strains

Labyrinthulid strain 00-Bat-05 was isolated from mangrove leaves at the Batan Bay mangrove area, the Philippines, using a double layer medium containing live diatom cells (Sakata and Iwamoto, 1995) which are described in details in **Chapter II**. Thraustochytrid strain HR-3 was provided by Dr. M. Hayashi, Faculty of Agriculture,

Miyazaki University, Japan and which was isolated from costal area of Hiroshima Prefecture, Japan. Chromosomal DNA of the strains was extracted and 18S rDNAs were amplified by PCR and sequenced by the standard method. A phylogenetic tree inferred from 18S rDNA nucleotide sequences was constructed by the neighbor-joining method (Saito and Nei, 1963).

Media and growth conditions

For the enzyme production, labyrinthulid strain 00-Bat-05 was grown at 25^{o}C for 168 h in 1 L of the ES (Provasoli's enrichment seawater) liquid medium in a 2 L Erlenmeyer flask with containing bacterial extract-egg yolk agar medium (NSBEY agar) according to Wahid *et al*. (2007). Thraustochytrid strain HR-3 was cultured at 25^{o}C for 96 h in 1 L of the medium containing 30 g glucose, 3 g yeast extract, 5 g polypeptone and 750 mL of artificial seawater (ASW, Herbst's formula composed of NaCl 30.0 g, KCl 7.0 g, $MgCl_2 \cdot 6H_2O$ 10.8 g, $MgSO_4 \cdot 7H_2O$ 5.4 g and $CaCl_2 \cdot 2H_2O$ 1.0 g per L, pH 7.6) in a flask on a reciprocal shaker (Taitec, NR-3, Japan) at 120 rpm.

Degradation or hydrolysis activity on bacterial dead cells, chemical compounds and nutrients

Digestion of dead cells of Vibrio parahaemolyticus by Labyrinthulids and Thraustochytrids:

Gram-negative bacteria *Vibrio parahaemolyticus* VPHK-46 was isolated from sea water of Kagoshima Bay and cultured in Z-CII liquid medium as described by Phyu Phyu Than *et al*. (2004). After 3 days incubation at 30˚C, bacterial cells were collected by centrifugation, 0.5 g wet weight of which was added to a nutrient media (0.5 g

polypeptone, 0.1 g yeast extract and 2.0 g NaCl in 100 mL distilled water, DW) and formed into agar plates (1.5% agar). Labyrinthulid cells (strain 00-Bat-05) and thraustochytrids cells (strain HR-3) were inoculated on the center of the bacterial dead cell plates and incubated at 25^{o}C. Bacteriolyses by labyrinthulid or thraustochytrid isolates were determined by the appearance of lytic zone around the labyrinthulid or thraustochytrid cells on the plates.

Hydrolysis activity on chemical compounds and nutrients by Labyrinthulids and Thraustochytrids:

Three chemical compounds starch, casein and chitin and one nutrient, egg yolk were used in this test. Starch 0.5 g, chitin 0.5 g and casein 2.0 g were separately added to a nutrient media (0.5 g polypeptone, 0.1 g yeast extract and 2.0 g NaCl in 100 mL DW) and formed into agar plates (1.5% agar). Egg yolk removed from a whole egg aseptically was mixed with equal volume of sterilized NSW and that solution was added the above media after sterilizing the media and formed into agar plates.

LAP enzyme preparation from the test organisms

Labyrinthulid strain 00-Bat-05

After incubation at the same conditions as above, the cells of labyrinthulid strain 00-Bat-05 collected from 8 L of liquid culture were washed with 50 mM Tris-HCl buffer (pH 7.6) and then suspended in 40 mL of 50 mM Tris-HCl buffer. The suspended cells were disrupted by sonication (4280S, Kaijo Denki, Japan) for 10 min, the cell debris was removed by centrifugation (12,000 x *g* for 20 min) and the supernatant was filtrated by 0.45 μm membrane filter (Advantec, Japan) and flow-through solution was used as a cell-free extract. Solid ammonium sulfate was added to the cell-free extract

solution to give 50% saturation. After 2 h, the precipitate was removed and the enzyme protein fraction was precipitated from the supernatant with ammonium sulfate (90% saturation) and collected by centrifugation. The precipitate was dissolved with 50 mM Tris buffer and dialyzed overnight against 50 mM Tris buffer. The dialyzed enzyme solution was loaded onto a Toyopearl DEAE-650M column (Tosoh, Japan) (2.0 x 22.0 cm) previously equilibrated with 50 mM Tris buffer and the proteinous materials were eluted with 50 mM Tris buffer and 500 mM NaCl using a linear gradient. The active fractions were pooled and concentrated by ultrafiltration with a YM-3 membrane (Amicon, USA). The enzyme solution was then applied to a Toyopearl HW-55F column (Tosoh, Japan) (2.0 x 22.0 cm) equilibrated with 50 mM Tris buffer containing 100 mM NaCl and eluted with the same buffer. The active enzyme fractions were combined, concentrated by ultrafiltration with a YM-3 membrane.

Thraustochytrid strain HR-3

After incubation at the same conditions as above, the cells of thraustochytrid strain HR-3 collected from 500 mL of culture broth were washed with 50 mM Tris-HCl buffer (pH 7.6) and then suspended in 50 mL of the same buffer. The suspended cells were disrupted by sonication and the cell debris was removed by centrifugation and the supernatant was filtrated by 0.45 μm membrane filter. Solid ammonium sulfate was added to the crude enzyme solution to give 60% saturation. After 4 h, the precipitate was collected by centrifugation, dissolved with 50 mM Tris buffer and dialyzed overnight against 50 mM Tris buffer. The dialyzed enzyme solution was loaded onto a Toyopearl Super Q-650M column (Tosoh, Japan) (2.0 x 22.0 cm) previously equilibrated with 50 mM Tris buffer and the proteinous materials were eluted with 50

mM Tris buffer and 500 mM NaCl using a linear gradient. The active fractions of LAP enzyme were pooled and concentrated by freeze drying. The freeze dried enzyme preparation was suspended in 50 mM Tris-HCl buffer and then applied to a Toyopearl HW-55F column (Tosoh, Japan) (2.0 x 70.0 cm) equilibrated with 50 mM Tris-HCl buffer containing 150 mM NaCl and the proteins were eluted with the same buffer. The active enzyme fractions were combined and concentrated by freeze drying.

Enzyme assay

Aminopeptidase activity was spectrophotometrically assayed by using L-leucine –*p*-nitroanilide (Leu-*p*-NA) as substrate described by Chien *et al.* (2002). The standard assay condition was as follows: 200 µL reaction mixture consisted of 20 µL of 10 mM L-leu-*p*-NA solution, 100 µL of 50 mM Tris-HCl buffer (pH 7.6) and 80 µL enzyme solution and incubated at 37^{o}C for 2 h. In addition, other *p*-Nitroanilide (*p*-NA) derivatives of L-amino acids were used at final concentration of 1 mM to measure the relative activity for LAP enzymes. Absorbance at 405 nm was determined by micro titer plate reader, MPR-A4I (Tosoh, Japan). All data are expressed as average values of duplicate experiments.

The protein concentration of enzyme preparations was determined by using the Micro BCA Protein Assay Kit (TaKaRa Bio, Japan) according to the supplier's manual with bovine serum albumin as the standard.

Polyacrylamaide gel electrophoresis

Native polyacrylamide gel electrophoresis (Native-PAGE) was performed with 7.5 % polyacrylamide gel as the separating gel according to the method described by

Chien *et al.* (2002). After native-PAGE, the gel was washed with deionized water for 2 min. The gel was incubated in LAP staining solution [100 mM sodium phosphate buffer (pH 5.8), 1 mM $CoCl_2$, 0.04% $_L$-leucyl-2-naphthylamide and 0.06% Fast Black K] in the dark at 37^oC until dark blue bands appear. The stained gel was washed with water then fixed in 7% acetic acid. Protein molecules in the gel also were visualized by staining with Silver staining kit (Silver Staining Kit, Protein, plus one, Amersham Biosciences, Sweden).

Effect of reaction temperature

By using the standard reaction mixture, enzymatic activity was determined at different temperatures between 4 and 80^oC for 2 h incubation.

Effect of heat treatment

Enzyme thermostability was determined after preincubation of the enzyme solution in 50 mM Tris-HCl buffer (pH 7.6) at various temperatures (4-80^oC) for 10 min and then the solution was cooled on ice for 5 min followed by the standard assay condition to measure residual enzyme activity.

Effect of reaction pH

Enzyme preparations were preincubated in 50 mM various buffer at 30^oC for 10 min. The optimum pH for enzyme reaction was examined under the standard assay condition using 50 mM buffer solutions with various pHs: sodium phosphate buffer (pH 6.0-7.0), Tris-HCl (pH 7.0-9.0), carbonate buffer (pH 9.0-11.0) and glycine buffer (pH 11.0-13.0).

Effect of inhibitors

The effect of *p*-chloromercuribenzoic acid (PCMB), phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), 1, 10-phenanthroline, iodoacetamide (IAA), bestatin and sodium dodecyl sulfate (SDS) on LAP activity was determined after preincubation with the enzyme and the chemicals (final concentration of 1 mM) in 50 mM Tris-HCl buffer (pH 7.6) for 30 min at 30^{o}C. The remaining activity was assayed under the standard condition.

Effect of metal ions

After preincubating the enzyme with various cations, chelating reagents or chemical reagents in 50 mM Tris-HCl buffer (pH 7.6) for 30 min at 30^{o}C. The remaining activity was assayed under the standard assay condition.

Bacteriolysis of bacterial dead cells by LAP

Aliquot (0.5 g, wet weight) of *Vibrio parahaemolyticus* dead cells collected was added into agar plates composed of 100 mL of 50 mM Tris-HCl buffer, pH 7.6 and 1.5% agar. Bacteriolytic activity was examined by using a paper disk containing 5 μL of LAP solution after 2 days incubated at 30^{o}C. Bacteriolysis by LAP enzyme was determined by the appearance of lytic zone around the paper disk on the agar plate. Only LAP from labyrinthulid was tested to know whether it related or not, to the bacteriolysis of the cells of *Labyrinthula* sp. strain 00-Bat-05.

Chemicals used

The synthetic chromogenic substrates, $_{L}$-leucine-*p*-nitroanilide (Leu-*p*-NA),

$_{L}$-alanine-*p*-nitroanilide (Ala-*p*-NA) and $_{L}$-glutamine-*p*-nitroanilide (Glu-*p*-NA) were obtained from Peptide Institute, INC. (Osaka, Japan) and $_{L}$-proline-*p*-nitroanilide (Pro-*p*-NA), $_{L}$-methionine-*p*-nitroanilide (Met-*p*-NA), $_{L}$-arginine-*p*-nitroanilide (Arg-*p*-NA) and $_{L}$-leucine-2-naphthylamide were from Sigma Chemical Co. (St. Louis, Mo., USA). The protease inhibitors used in this study were purchased from Sigma and Nakarai Tesuque (Kyoto, Japan).

Results

Phylogenetic analysis of strains 00-Bat-05 and HR-3

Figures III-1A and 1B show cell morphology of labyrinthulid strain 00-Bat-05 and thraustochytrid strain HR-3, respectively. The individual cells of labyrinthulid 00-Bat-05 (Figure III-1A) are typically spindle form with 2.5 - 5.0 μm wide by 8.0 -12.0 μm long. On the other hand, those of thraustochtrid HR-3 (Figure III-1B) are round form with 10.0 - 15.0 μm in diameter, which contain numerous granules in the cytoplasm. The 18S rDNA sequence of strain 00-Bat-05 (GenBank accession no. AB290459) shows 93% homology with that of *Labyrinthula* sp. AN-1565 (AB022105), while strain HR-3 has 99% homology with Thraustochytriidae sp. NIOS-1 haplotype NIOS1-D00-1 (AY705769). A phylogenetic tree constructed by the neighbor-joining method is shown in Figure III-2.

Degradation and hydrolysis activity on bacterial dead cells, chemical compounds and nutrients

As shown in Figure III-3A, labyrinthulid strain 00-Bat-05 can easily lyse the bacterial dead cells but the thraustochytrid HR-3 strain could not degrade *V. parahaemolyticus* cells (Figure III-3B). The tests result of hydrolysis of chemical compounds and nutrient are given in Table III-1. It was found that labyrinthulid strain 00-Bat-05 could hydrolyze starch (Figure III-4A) and casein (Figure III-5A) but could not chitin and egg yolk. Thraustochytrid strain HR-3 could not digest these chemical compounds but it could lyse only egg yolk outside of their colony (Figure III-6B) and could grow in presence of chemical compounds.

Enzyme preparation

Partially purified enzyme preparations were obtained after ion-exchange (Figure III-7) and gel filtration chromatography described in Materials and Methods. On gel filtration chromatography (Figure III-8A), active peak of LAP enzyme from 00-Bat-05 strain was detected between fraction number 11 and 15. Pooled active fractions eluted from gel filtration were concentrated and applied to native-PAGE. Native-PAGE and activity staining of LAP enzyme showed that a major protein band possessed LAP activity (Figure III-8B). Purification steps of LAP from 00-Bat-05 strain are summarized in Table III-2. The specific activity of the purified enzyme was about 60-fold higher than that of cell-free extract solution after ultrasonic treatment of the cells. On the other hand, one active fraction of LAP from HR-3 strain was isolated although it still included several protein molecules as judged from polyacrylamide gel electrophoresis (PAGE). The partially purified enzymes from each strain were used for characterization of LAP activity.

Characterization of LAP enzymes

Optimal temperature

Under the conditions described in Materials and Methods, LAP activities of both strains were at their maxima at 37°C as shown in Figure III-9. More than 50% of maximum activity was found between 15 to 45°C in both strains. More than 80% activity of 00-Bat-05 LAP was remaining after heat treatment at 60°C for 10 min, while HR-3 LAP enzyme was almost inactivated with heat treatment at the same temperature (Figure III-10).

Optimal pH

As shown in Figure III-11, the optimum pH was 8.0 for 00-Bat-05 LAP and 7.0-8.0 for HR-3 LAP. However, the enzyme activity of 00-Bat-05 strain was found to be considerably low at pH 9.0, suggesting that it was inactivated at pH 9.0 with some reasons.

Effect of inhibitors

Effect of various inhibitor substances on LAP activity is shown in Table III-2. In strain 00-Bat-05, the LAP activity was strongly inhibited by bestatin, which is generally known to be an inhibitor of aminopeptidase (Table III-3). Strong inhibition was also observed in the presence of EDTA, SDS, PCMB, DTT, and 1,10-phenanthroline. EDTA was known to be a metal-chelating agent, and PCMB to be a SH inhibitor. On the other hand, LAP activity from HR-3 strain was inhibited by SDS, PCMB, 1,10-phenanthroline, and bestatin but activated by EDTA and DTT.

Effect of metal ions

The effect of several metal ions on enzyme activity is shown in Table III-4. Among the metal ions tested in LAP of strain 00-Bat-05, enzyme activity was strongly inhibited by Zn^{2+}, Hg^{2+} and Ni^{2+} and moderately inhibited by Mn^{2+}, Mg^{2+}, Cu^{2+}, $Ba^{2+}$and K^{+}. However, Co^{2+} strongly activated the enzyme activity from strain 00-Bat-05. On the other hand, the LAP activity of strain HR-3 was strongly inhibited by Zn^{2+}, Hg^{2+} and Ni^{2+}, and slightly inhibited by Co^{2+}. Metal ions such as Na^{+}, K^{+}, and Mg^{2+} stimulated the LAP activity of strain HR-3.

Substrate specificity

The ability of the enzyme to catalyze the hydrolysis of various *p*-NA derivatives is shown in Table III-5. In strain 00-Bat-05, Leu-*p*-NA was most efficiently hydrolyzed by LAP. Met-*p*-NA, Ala-*p*-NA and Arg-*p*-NA were also to be considerably hydrolyzed, while Pro-*p*-NA and Glu- *p*-NA were practically resistant to the action of the enzyme. In strain HR-3, Ala-*p*-NA was most efficiently hydrolyzed by the enzyme and Leu-*p*-NA, Met-*p*-NA and Arg-*p*-NA were good substrate for LAP. On the other hand, Pro-*p*-NA and Glu-*p*-NA were not optimal to the enzyme activity from stain HR-3.

Effect of LAP on dead cells of *Vibrio parahaemolyticus*

Partially purified LAP enzyme, from strain 00-Bat-05, showed the bacteriolysis activity against the dead cells of *Vibrio parahaemolyticus* in Figure III-12.

Discussion

Both labyrinthulids and thraustochytrids have a role in decomposing plant matters in coastal environments and their degrading enzymes are involved in this process (Porter, 1990). Labyrinthulid strain 00-Bat-05 hydrolyzed the starch and casein but not chitin. Fujisawa (1998) also found the same phenomenon in *Labyrinthula* strain L95-1. L95-1 had shown positive results in starch and casein and negative results in chitin and try-n-butyrin.

This study also compared the enzymological properties of LAP enzymes from labyrinthulid and thraustochytrid strains isolated from marine environments. The LAP enzyme of strain 00-Bat-05 had an optimal temperature of 37^{o}C but 80 % of its maximal activity remained even after heat treatment at 60^{o}C for 10 min. On the other hand, the optimal temperature for the LAP activity of strain HR-3 was 37^{o}C and the enzyme was inactivated completely with heat treatment at 60^{o}C for 10 min. LAP enzyme of 00-Bat-05 was strongly inhibited by DTT, suggesting the presence of a disulfide linkage which is required to maintain its active conformation and which is similar to thermotolerant LAP (Deejing *et al*., 2005). The thermostability of the enzyme is therefore caused by the presence of a disulfide linkage which is not seen in HR-3 LAP due to any inhibition by DTT. The thermostability of aminopeptidase is due to disulfide bond formation (Chen *et al*., 1997).

The optimum pH of LAP from strain 00-Bat-05 was 8.0 and high activity was still detectable at pH 10.0 and 11.0. However, the activity was relatively low under neutral conditions (pH 7.0). The HR-3 LAP had a broad pH range from 6.0 to 11.0 and the activity was still found about 50 % at pH 11.0 and 12.0. These results indicated that

both enzymes have optimal activity at alkaline region similar to other aminopeptidases (Lee *et al*., 1998 and Chien *et al*., 2002).

The LAP activities from strains 00-Bat-05 and HR-3 were inhibited by PCMB, suggesting both LAP enzymes to be SH enzymes. Metal-chelating agent, EDTA inhibited the 00-Bat-05 LAP, while it inhibited a little bit the HR-3 enzyme. Both enzymes were inhibited by bestatin, which were proven that both enzymes are aminopeptidase. The LAP activity from 00-Bat-05 was inhibited by 1,10-phenanthroline and EDTA and similar results were found in LAP from *Geobacillus thermoleovorans* (Deejing *et al*., 2005). Most of LAP enzymes from bacteria and fungi were reported to be inhibited by metal-chelating agents and to be largely restored by divalent cations such as Zn^{2+} and Co^{2+} (Lee *et al*., 1998; Chien *et al*., 2002). The LAP activity of strain 00-Bat-05 was inhibited by Zn^{2+}, and stimulated by Co^{2+}, while the enzyme activity of strain HR-3 was slightly inhibited by Co^{2+} and strongly inhibited by Zn^{2+}. The enzyme of strain 00-Bat-05 was inhibited by K^{+} but the enzyme of strain HR-3 was distinctively activated by Na^{+} and K^{+}. These results indicate that effect of metal ions on LAP activity varies from organism to organism.

As substrates, Leu-*p*-NA was best for the LAP enzyme of strain 00-Bat-05 LAP, followed by Met-*p*-NA (51 %), Ala-*p*-NA (21 %) and Arg-*p*-NA (20 %) and negligible activity was detected for Glu-*p*-NA and Pro-*p*-NA. On the other hand, the enzyme of strain HR-3 showed high activity for Leu-*p*-NA (100 %), Ala-*p*-NA (347 %), Met-*p*-NA (109 %) and Arg-*p*-NA (125 %) and slight activity for Glu-*p*-NA and Pro-*p*-NA.

Partially purified LAP enzyme, from strain 00-Bat-05, showed the bacteriolysis activity against the dead cells of *Vibrio parahaemolyticus* which phenomenon was also found by the cells of *Labyrinthula* sp. strain 00-Bat-05.

This is the first report on the isolation and enzymological properties of LAP enzymes from labyrinthulid and thraustochytrid strains. The information obtained in this study will help to reveal the ecological roles in degradation process of plant materials and microbial cells in marine environments and physiological response of these organisms as live food of aquaculture animals.

Table III-1. Comparable table of hydrolysis of chemicals and nutrient between labyrinthulid and thraustochytrid strains

Chemical compounds and nutrient	Labyrinthulid Strain 00-Bat-05	Thraustochytrid Strain HR-3
Starch	+	-
Casein	+	-
Chitin	-	-
Egg yolk	-	+

Table III-2. Purification of LAP from strain 00-Bat-05

Purification	Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification (fold)	Yield %
Cell-free extract	40	24000	1184	20.3	1	100
$(NH_4)_2SO_4$	10	10000	96	104.2	5.1	41.7
Toyopearl DEAE-650M	4	8600	10	860.0	42.4	35.8
Toyopearl HW-55F	2	4946	4	1236.7	61.0	20.6

* One unit of LAP activity was defined as the amount of enzyme that cause an incremental change of 0.01 absorbance per hr incubation at 37^{o}C.

Table III-3. Effect of chemical reagents on LAP activity from test strains

Chemical reagents*	Relative activity (%)	
	00-Bat-05	HR-3
No addition	100.0	100.0
EDTA	40.2	88.0
PCMB	13.6	10.0
DTT	16.4	78.0
IAA	132.4	91.4
1,10-phenanthroline	12.8	16.0
PMSF	106.8	89.0
SDS	3.4	17.5
Bestatin	5.2	35.7

* Each chemical reagent was added to a final concentration of 1 mM in the reaction mixture.

Table III-4. Effect of metal ions on LAP activity from test strains

Metal ions*	Relative activity (%)	
	00-Bat-05	HR-3
No addition	100.0	100.0
NaCl	108.5	187.8
KCl	55.8	155.7
$MnCl_2$	76.0	104.3
$MgCl_2$	62.8	178.8
$ZnCl_2$	31.0	29.7
$CuSO_4$	52.7	100.0
$CaCl_2$	79.9	99.5
$HgCl_2$	30.2	23.1
$NiCl_2$	12.9	46.7
$CoSO_4$	298.6	75.0
$BaCO_3$	55.5	87.3

* Each salt was added to a final concentration of 1 mM in the reaction mixture.

Table III-5. Hydrolytic activities of LAP from test strains on various substrates

Substrate*	Relative activity (%)	
	00-Bat-05	HR-3
Leu-*p*-NA	100.0	100.0
Ala-*p*-NA	21.0	347.0
Pro-*p*-NA	1.3	34.2
Met-*p*-NA	51.0	109.4
Arg-*p*-NA	20.1	125.0
Glu-*p*-NA	5.2	22.7

* All substrates were used at a final concentration of 1 mM under the standard assay conditions.

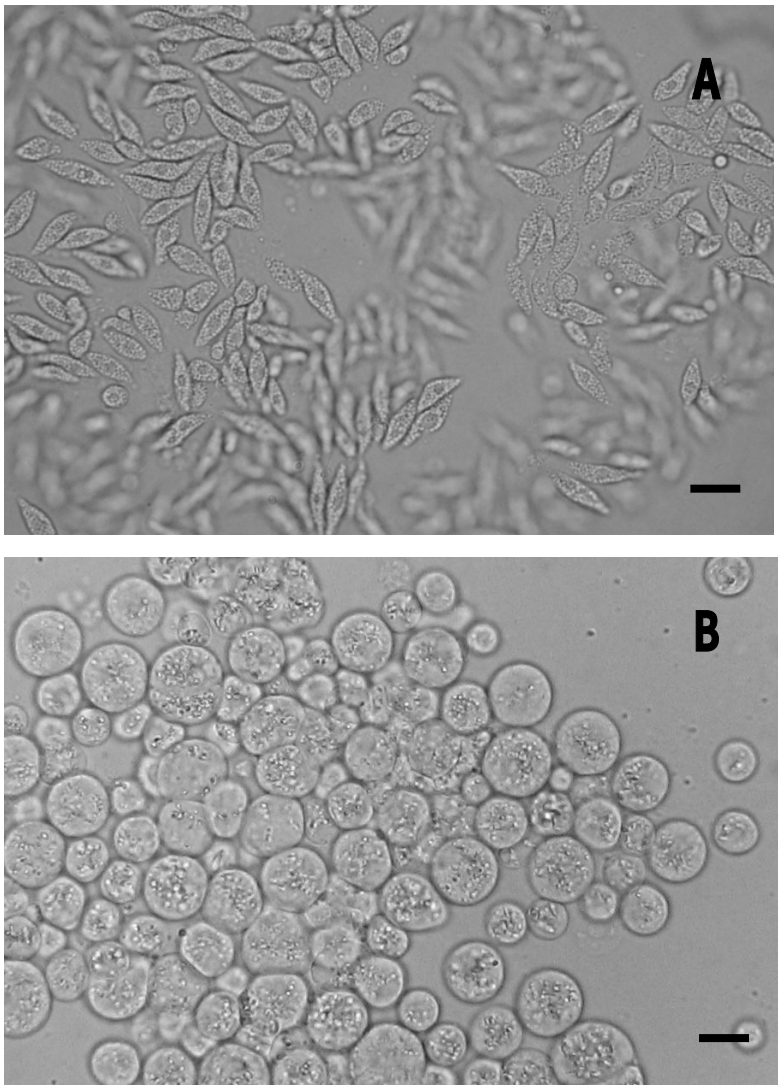


Figure III-1. Photograph of vegetative cells of labyrinthulid strain 00-Bat-05 (A) and thraustochytrid strain HR-3 (B). Scale bars indicate 10 μm.

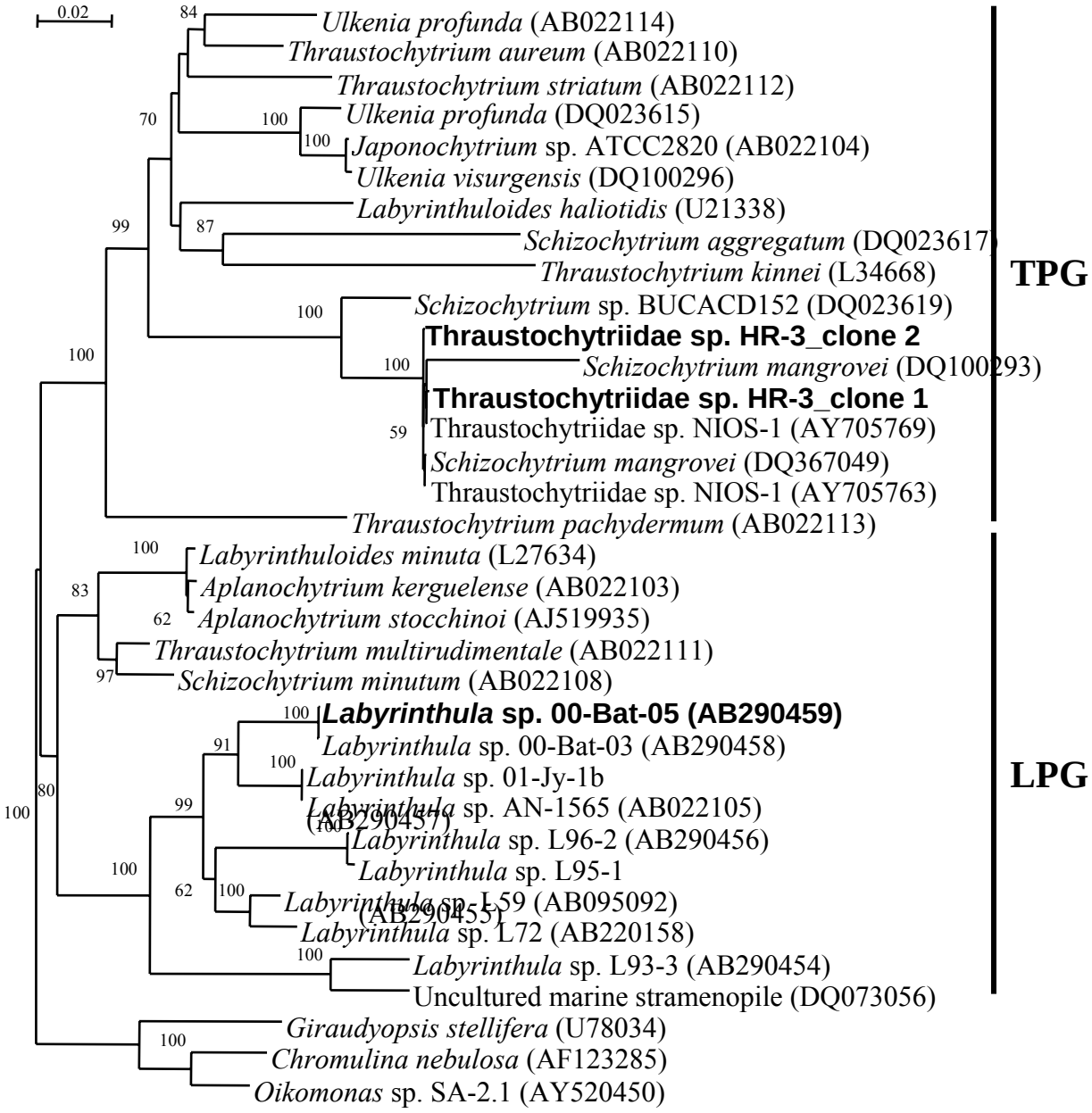


Figure III-2. Phylogenetic tree inferred from 18S rDNA of labyrinthulid sp. 00-Bat-05 and thraustochytrid sp. HR-3 by the neighbour-joining method. Bootstrap values greater than 50% are shown above the branches. A scale bar indicates genetic distance. LPG, labyrinthulid phylogenetic group; TPG, thraustochytrid phylogenetic group.

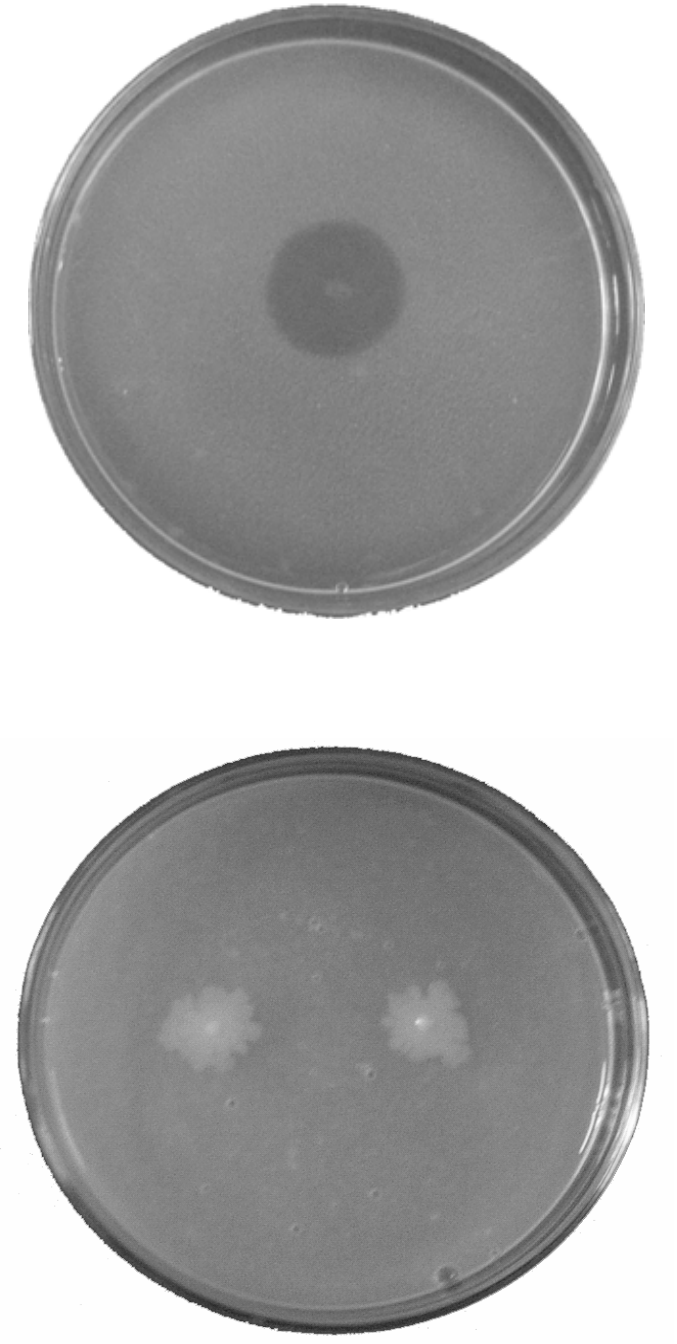
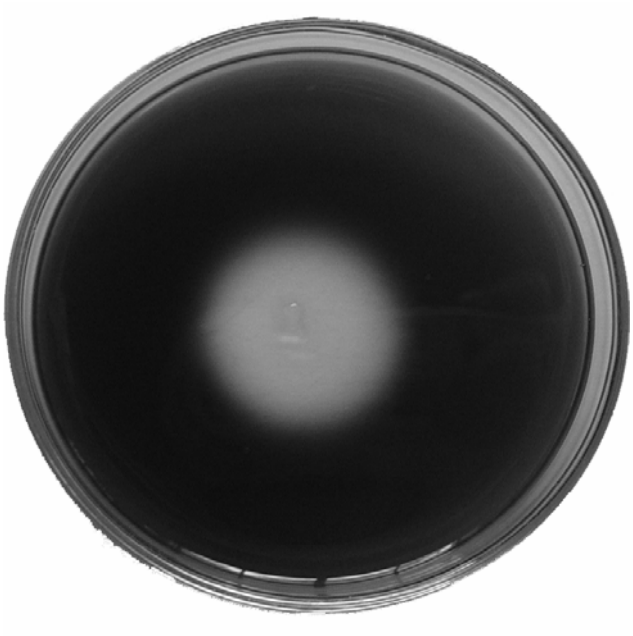


Figure III-3. Bacteriolysis of labyrinthulid sp. strain 00-Bat-05 (A) and thraustochytrid sp. strain HR-3 (B) on dead cells of *Vibrio parahaemolyticus*. A clear lytic zone was formed by *Labyrinthula* sp. strain 00-Bat-05 and HR-3 strain was grown well on dead cells of *V. parahaemolyticus.*



A

B

Figure III-4. Test result of starch hydrolysis between labyrinthulid sp. strain 00-Bat-05 (A) and thraustochytrid sp. strain HR-3 (B). In A, a clear hydrolysis zone is visible.

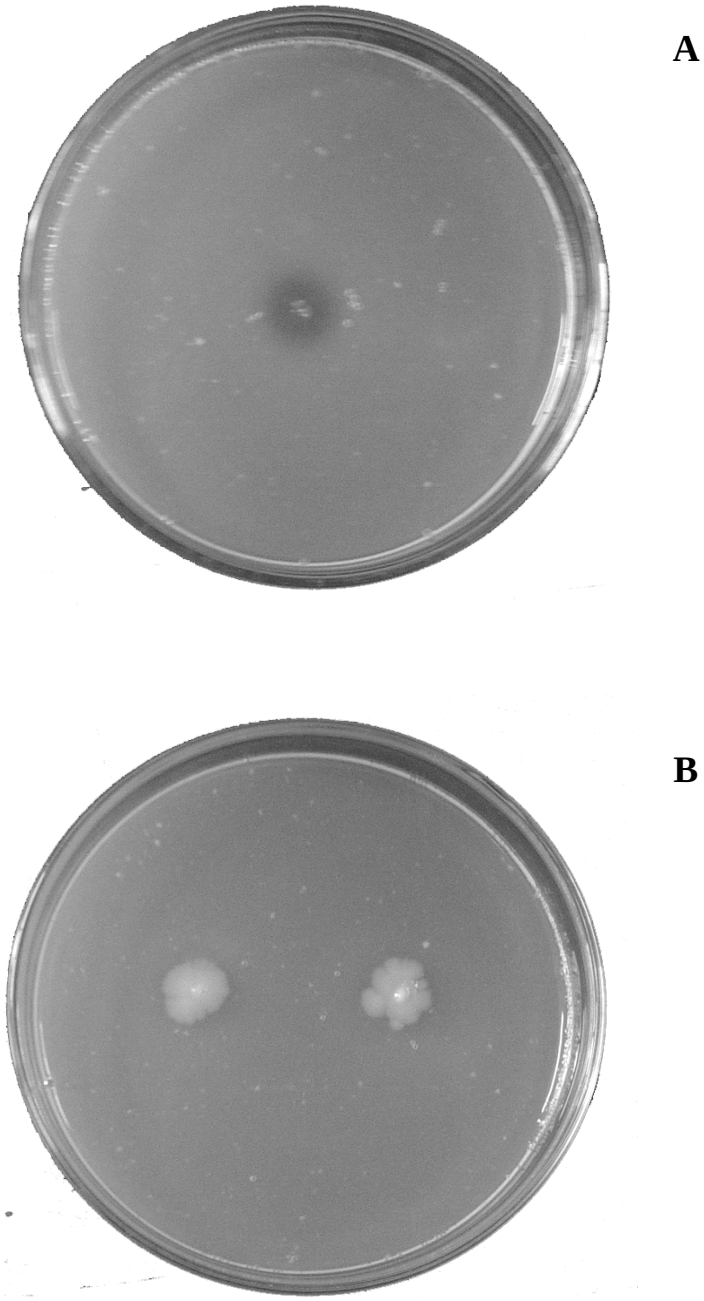
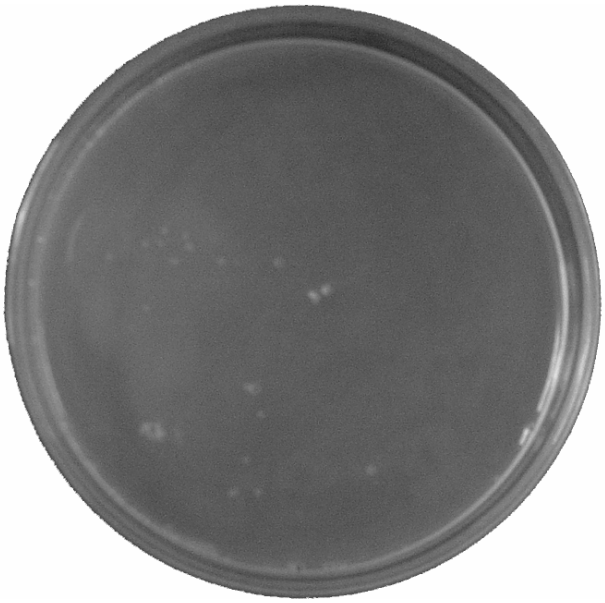
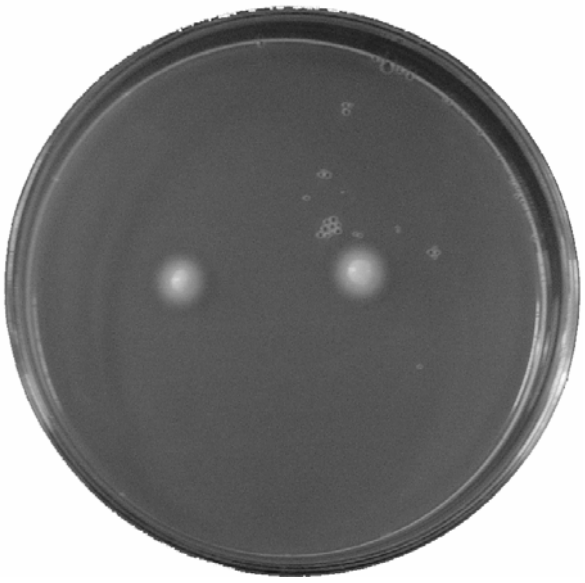


Figure III-5. Hydrolysis test of casein between labyrinthulid sp. strain 00-Bat-05 (A) and thraustochytrid sp. strain HR-3 (B). Casein hydrolysis was observed by *Labyrinthula* sp. strain 00-Bat-05 in A.



A



B

Figure III-6. Egg yolk degradation test between labyrinthulid sp. strain 00-Bat-05 (A) and thraustochytrid sp. strain HR-3 (B). Weak lytic circles were observed just outside of the HR-3 strain's colonies.

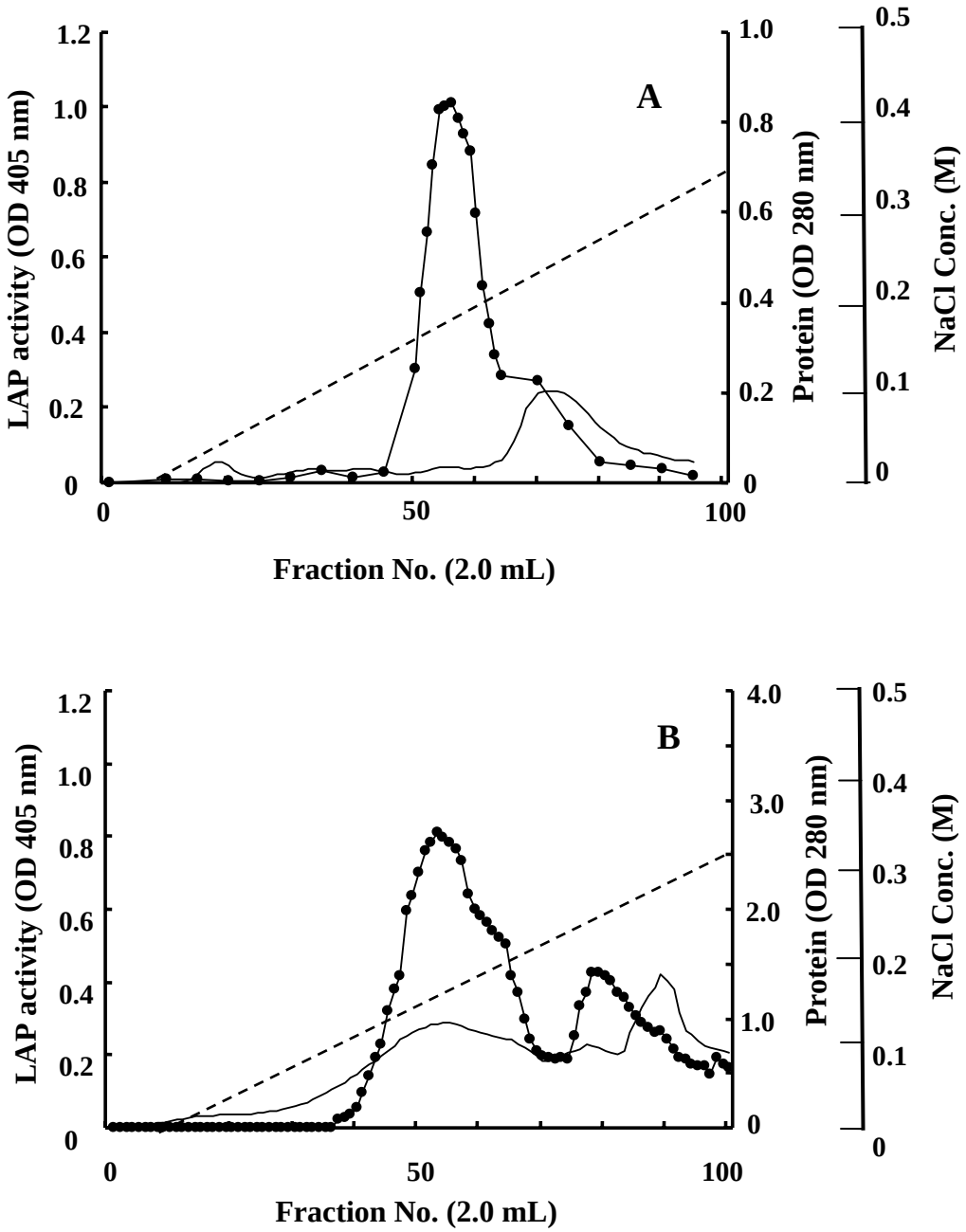


Figure III-7. Chromatographic profiles of LAP enzymes from test strains. A, Toyopearl DEAE-650M chromatogram of LAP from strain 00-Bat-05; B, Toyopearl Super Q-650M chromatogram of LAP from strain HR-3. ●, LAP activity (OD at 405 nm); —, protein concentration (OD at 280 nm); ---, NaCl concentration (M).

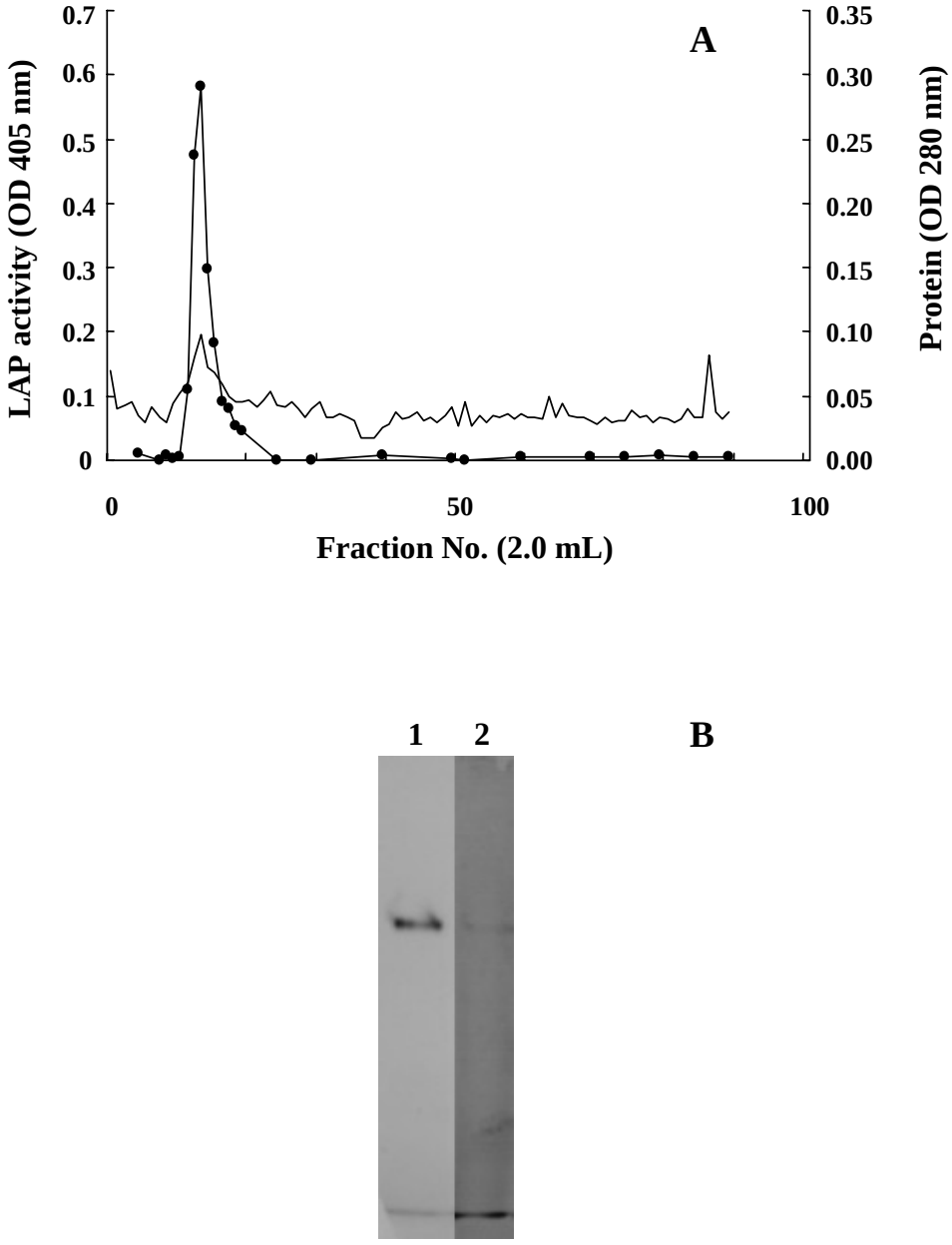


Figure III-8. In A, Gel filtration chromatography profile of LAP from strain 00-Bat-05; ●, LAP activity (OD at 405 nm) and —, protein concentration (OD at 280 nm). In B (1), activity staining of LAP enzyme from strain 00-Bat-05 and representative protein band is visible by silver staining after native PAGE in B (2).

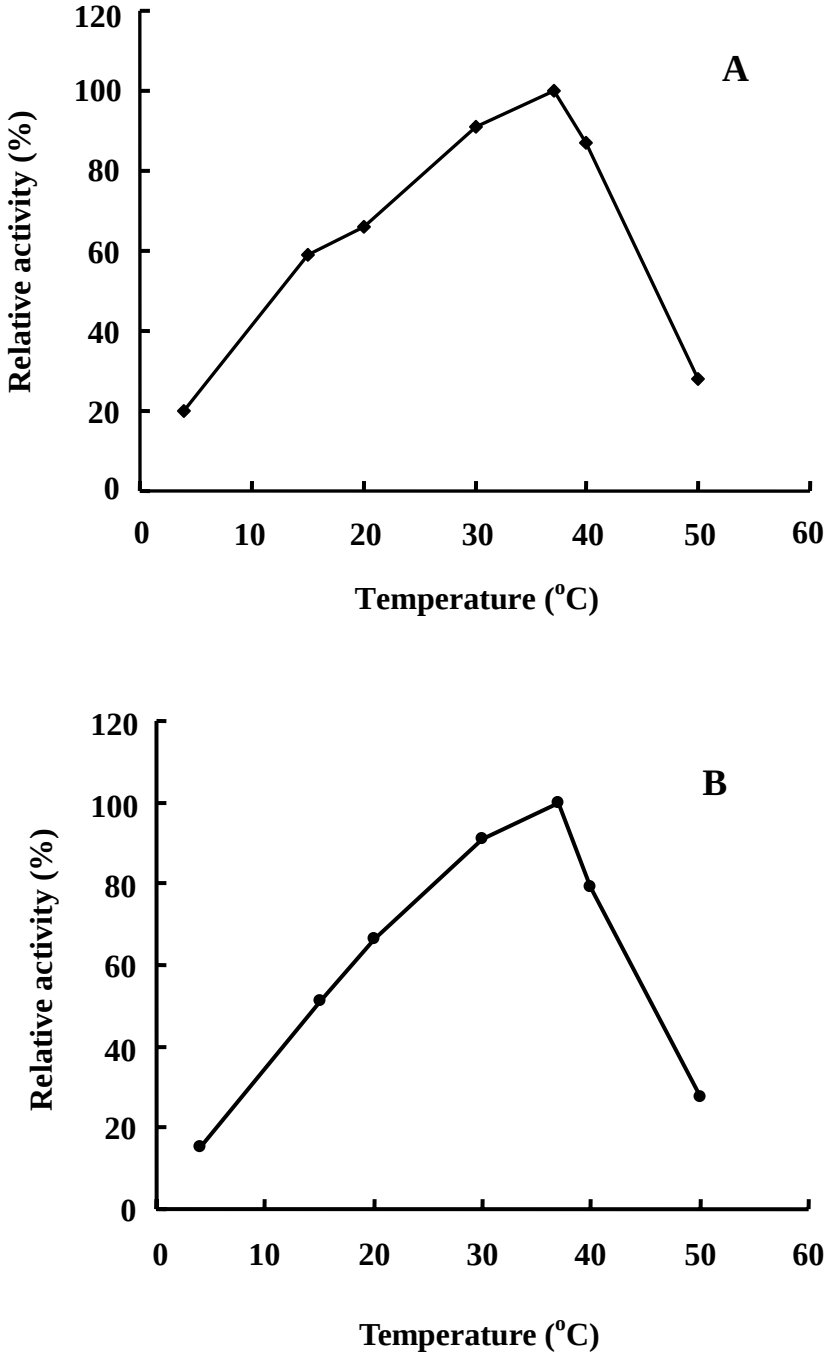


Figure III-9. Effect of reaction temperature on enzyme activity from strains 00-Bat-05 (A) and HR-3 (B). Enzyme activity was measured at various temperatures in 50 mM Tris-HCl buffer (pH 7.6). Activities are expressed relative to the maximum value.

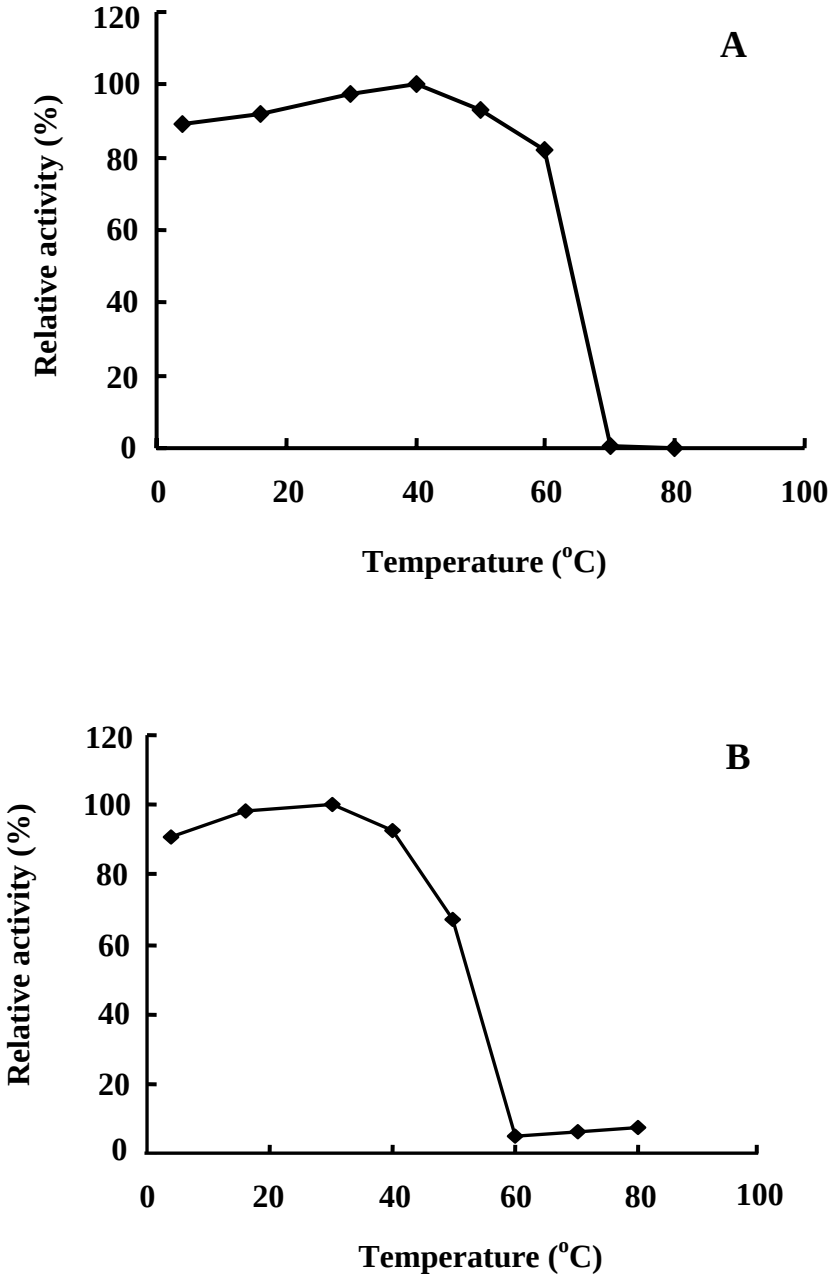


Figure III-10. Effect of heat treatment on enzyme stability of strains 00-Bat-05 (A) and HR-3 (B). Enzyme preparations were heated in 50 mM Tris-HCl buffer (pH 7.6) at various temperatures for 10 min

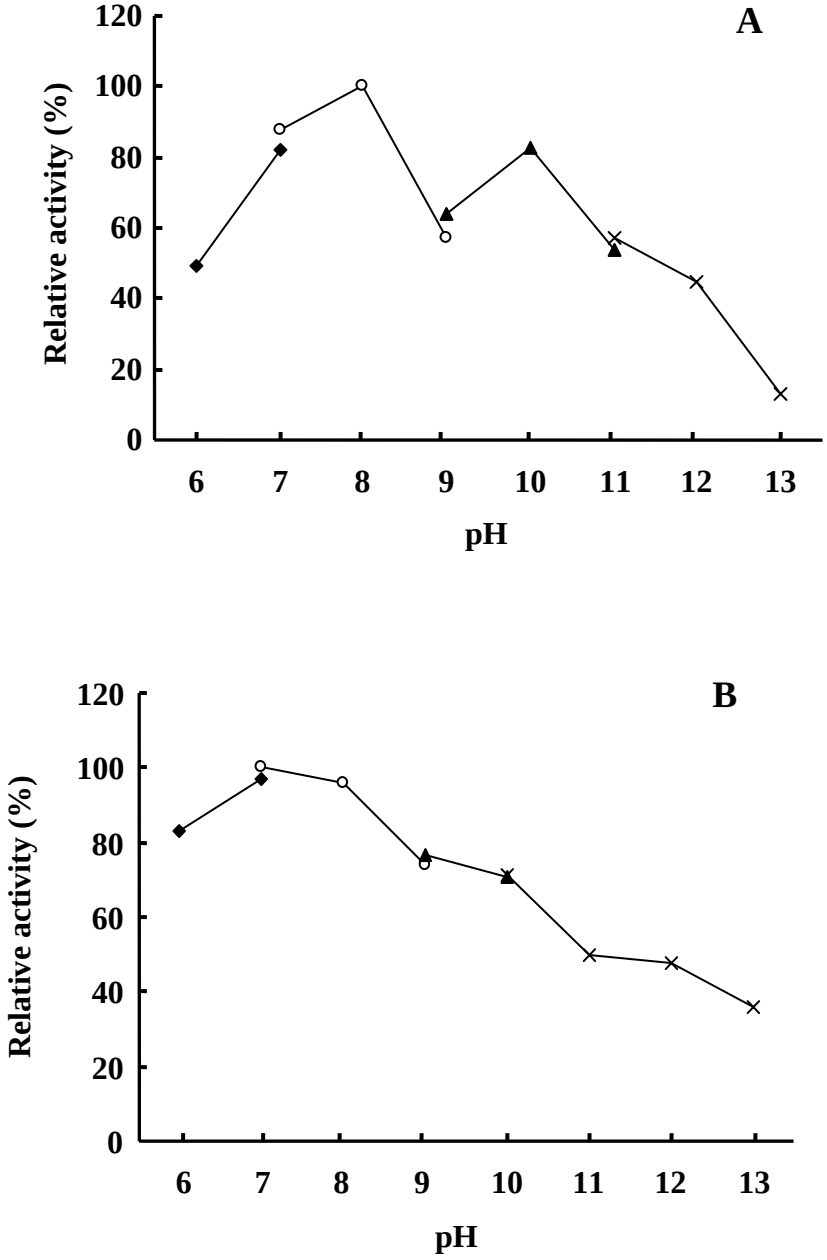


Figure III-11. Effect of pH on enzyme activity of strains 00-Bat-05 (A) and HR-3 (B). Enzyme preparations were preincubated in 50 mM various pH buffer at 30°C for 10 min. ◆, sodium phosphate buffer; ○, Tris-HCl buffer; ▲ , carbonate buffer; ×, glycine-NaOH buffer.

Figure III-12. Bacteriolysis test of LAP from labyrinthulid strain 00-Bat-05 on dead cells of *V. parahaemolyticus*. Lytic zones were observed after 24 h (A) and 48 h (B) incubation at 30°C.

Chapter IV

General Conclusion

Thirteen strains of labyrinthulids were isolated from the coastal area of Kagoshima Bay, Kagoshima Prefecture, Japan and from Batan Bay, Panay Island, the Philippines by using diatom double-layer agar plates. Among 11 strains collected from Kagoshima Bay, two were originated from coastal seawater, three from green algae (*Ulva* spp.), two from brown algae (*Sargassum* spp.) and four from submerged mangrove leaves (*Kandelia* sp.). The other two strains were isolated from submerged mangrove leaves in Batan Bay, the Philippines. The cells of labyrinthulid isolates grew axenically on the bacterial dead cells and extract plus egg yolk agar medium (NSBEY agar) and obtained large amount of growing cells of marine *Labyrinthula* spp. Three representative isolates were demonstrated to belong to the labyrinthulid phylogenetic group (LPG) based on 18S rDNA sequence analysis. In the hydrolysis test of four representative labyrinthulid isolates, it was found that they could hydrolyse macromolecule compounds like starch and even two isolates could hydrolyse casein but none of them could hydrolyse complex chemical compounds like chitin and try-n-butyrin. Ten enzymes were detected by using API-ZYM Kit in four representative isolates. Three enzymes including alkaline phosphatase, leucine arylamidase and valine arylamidase were found in higher level in API-ZYM tests. As examined their bacteriolytic activity of labyrinthulid isolates, it was found that they could lyse only the dead cells of Gram-negative bacteria and not those of Gram-positive bacteria during incubation in both agar and liquid media. The optimum temperature range for their bacteriolysis was from 25 to 31^{o}C. Respiratory inhibitors had repressed bacteriolysis activity but no impact on viability was found in *Labyrinthula* cells of strain 00-Bat-05. Orange carotenoid pigment(s) was accumulated during stationary growth phase of a strain 00–Bat-05, Philippine isolate, cultured in an

L-shape tube containing a bacterial dead cell suspension and concomitantly rapid cell movement of developing zoospores was observed

Leucine aminopeptidases (LAPs) from marine labyrinthulid strain 00-Bat-05 and thraustochytrid strain HR-3 cells were partially purified and characterized by enzymological properties. The optimum temperature of LAPs from both strains was 37^{o}C. The thermostability of 00-Bat-05 LAP was indicated by having 80% of maximum activity after heat treatment at 60^{o}C for 10 min, while LAP activity of strain HR-3 was completely inactivated at 60^{o}C. LAP activities from both strains were high at near pH 8.0. Both LAPs were inactivated by 1,10-phenanthroline, *p*-chloromercuribenzoic acid (PCMB), bestatin and sodium dodecyl sulphate (SDS), suggesting that both are SH-aminopeptidase.. Enzyme activity of LAP from strain 00-Bat-05 was stimulated by Co^{2+} and inhibited by Zn^{2+}, while that from HR-3 was inhibited by Co^{2+} and Zn^{2+}. LAP of 00-Bat-05 had a high specificity for L-leucine-*p*-nitroanilide but HR-3 enzyme showed relatively broad specificity for *p*-nitroanilide derivatives of L-amino acids.

Partially purified LAP enzyme, from strain 00-Bat-05, showed the bacteriolysis activity against the dead cells of *Vibrio parahaemolyticus* which phenomenon was also found by the cells of *Labyrinthula* sp. strain 00-Bat-05.

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