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CLONING, SEQUENCING AND TRANSCRIPTIONAL ANALYSIS OF UBIQUITIN GENE FROM *ALEXANDRIUM CATENELLA* UNDER DIFFERENT NUTRIENT CONDITIONS

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Abstract

Ubiquitin is a small conserved protein ubiquitous in all eukaryotic organisms. It plays a critical role in intracellular protein turnover, being the "tag" that marks proteins for proteolysis. Beside proteolysis, it plays vital role in many cellular processes where protein actively needs to be modified or relocated. Sequence of ubiquitin selected from the differentially expressed gene library of NGS data of *Alexandrium catenella* was compared with the NCBI database for homologous sequences by blast. Primers were designed for amplification of partial fragment and gene expression analysis using real-time PCR in cells cultured at different nutritional conditions. Dino SL primer reported in the preceding studies was utilized to obtain a complete 5' end. A full-length cDNA of ubiquitin gene was obtained upon the assembly of the amplified products. Conserved domains of ubiquitin including the hydrophobic surface conserved amino acid regions and Ulp1-Smt3 interaction sites were observed in the cloned sequence. Phylogenetic tree constructed from the deduced amino acid sequence revealed two distinct clades representing ubiquitin sequences from dinoflagellates. The amino acid sequence of *A. catenella* has shown to possess 100% identity at conserved regions with another dinoflagellate, *Pfiesteria piscicida*. The result of qRT-PCR indicated that the gene was significantly upregulated under all tested conditions. We speculate that under high nitrogen (HN) and high nitrogen phosphorous condition (HNP), the up-regulation of genes is due to rapid growth of the algae where proteins are also produced in excess amount. On the other hand, at low nitrogen and phosphorous conditions (LNP), the algae were under the effects of reactive oxygen species and as a result, the activation of ubiquitin-proteasome system is triggered that results in upregulation of ubiquitin family proteins to clear off the damaged proteins. The phenomenon is well supported by previous reports in other organisms. This study provides an important first look into the relationship between nutrient supply and expression analysis of ubiquitin in dinoflagellates.

Key words: Dinoflagellates, Gene expression, Phytoplankton, RT-Qpcr.

Introduction

Ubiquitin is a highly conserved (Izquierdo *et al.*, 1984; Özkaynak *et al.*, 1984; Wiborg *et al.*, 1985; Arribas *et al.*, 1986) small protein present in all eukaryotes, consisting of 76 amino acids, present both as a free polypeptide and covalently joined to various cytoplasmic, nuclear and cell surface proteins. The ubiquitin system is a major controller of cellular physiology and play central role in protein degradation processes, signaling pathways (Clague *et al.*, 2015), as a modifier of chromatin structure during its conjugation to histones H2A and H2B (Levinger & Varshavsky, 1982; Thorne *et al.*, 1987) which may facilitate transcription and DNA repair (Levinger & Varshavsky, 1982), receptor control by endoytosis (Christensen *et al.*, 1992; Heegaard *et al.*, 1995; Okazaki *et al.*, 2000), ATP-dependent non-lysosomal protein degradation pathway (Ciechanover *et al.*, 1984; Hershko *et al.*, 1984), and also its conjugation system serve as a sensor of the heat shock response (Parag *et al.*, 1987). Regardless of its discovery as a protein that appears to show up ubiquitously, at least in eukaryotic cells, researcher are only commencing to unravel all of the cellular functions that entail ubiquitin (Keith, 2012).

Ubiquitin genes are categorized into two classes: The one that binds to protein as a monomer are called monomeric (Keith, 2012), while the polymeric binds the proteins in long chains that bend or branch (Binet *et al.*, 1991). Monomeric ubiquitin is a 76 codons protein (consists of 228 nucleotides). An additional C-terminal sequence is also present that encodes a ribosomal protein (Garbarino & Belknap, 1994). On the other hand, several direct head-to-tail repeats of the ubiquitin coding units are present in polyubiquitins (also known as polymeric genes) (Ozkaynak *et al.*, 1987; Finley *et al.*, 1989). Ubiquitins are also termed as little messengers because they work as a molecular tag and has decisive role in ubiquitin-proteasome proteolytic pathway. During this course of action, ubiquitin binds to a lysine present in the target protein through isopeptide bond by a continual process to form a polyubiquitin chain, which is recognized by 26S proteasome and marked protein is then degraded within the core of the proteasome (Marinovic *et al.*, 2000). Accessory proteins (E1, E2, E3) aids in selection of proteins to be tagged and recruit ligases to join ubiquitin mononmers into a chain. Membrane proteins are also tagged by ubiquitin that are afterwards pinched inside the endosomal vesicles and are transported to the lysosome for digestion (Keith, 2012).

The ubiquitin pathway has been studied in a number of organisms including plant, animals, fungi and algae. In *Saccharomyces cerevisiae*, it has been comprehensively characterized and was found to be significant in key processes including mediating cell differentiation and in responses to environmental stress (Hilt & Wolf, 1995). Mounting evidences are found in plants, animals and other organisms showing participation of ubiquitin pathway in growth and stress responses (Belknap & Garbarino, 1996; Laney & Hochstrasser, 2004). In *Cyanidazzioschyzon merolae*, a red alga, E3 ubiquitin ligase is shown to ubiquitylate the relevant cyclin and induce its degradation (Kobayashi *et al.*, 2011). In Antarctic seaweed, *Plocamium cartilagineum*, significant induction of ubiquitin mRNA was detected after it was exposed to heat shock (Vayda & Yuan, 1994). Upon heat treatment at greater than 40°C of unicellular green

microalgae, *Chlamydomonas reinhardtii*, radical raise in ubiquitinated proteins was observed (Vayda & Yuan, 1994). A previous report on *C. reinhardtii* also supports the response of ubiquitin to heat stress along with light and its role in chloroplast (Wettern *et al.*, 1990).

A. catenella is one of the most studied marine dinoflagellate due to its ecological, toxicological and economic significance. It is known to produce saxitoxins, a strong neurotoxins that results in paralytic shellfish poisoning (John *et al.*, 2014). A study reported that during initiation phase of *A. catenella*, almost 25% of the identified genes signified the enzymes and proteins that contribute in a range of cellular regulatory mechanisms that may characterize proliferating cells, e.g., control of the cell cycle and division, regulation of transcription, translation and posttranslational protein modifications, signaling, intracellular trafficking, and transport. High protein yield is implicated in the dynamics of cellular and metabolic processes as suggested by the expression of genes involved in protein degradation by ubiquitination and proteasome formation (Toulza *et al.*, 2010).

Conversely, Ubiquitin pathway during growth and developmental stages of *A. catenella* has received very little attention. Therefore, the present study was aimed to identify and characterize ubiquitin gene in *A. catenella* grown under diverse nutritional conditions. Ubiquitin was found to be differentially expressed in transcriptomics *de novo* assembly sequencing and analysis of *A. catenella* (Zhang *et al.*, 2014). Here we also present the expression analysis and the information given here can lead us towards a better understanding of response of ubiquitin gene expression towards growth under different nutritional conditions.

Materials and Methods

Algal culture: The culture of *A. catenella* used in this study was obtained from Key Lab of Marine Genetics and Breeding, Ministry of Education of China, College of Marine Life Sciences. Stock cultures were grown in f/2 medium (silicate free) under normal growth conditions at 20±1°C with a 12:12 h light: dark photocycle (Guillard, 1975) and a photon flux density of 30-35µmolm $^{-2}$ s $^{-1}$ provided by cool white fluorescent lights.

Initially approximately 5000 cells per ml were inoculated in each flask of growth medium in triplicates, synchronized by keeping in dark for 48 hours and were then exposed to light for activation. Cell count was done on alternative days starting from day 01 in 1 ml of sample preserved in Lugols Iodine using hemocytometer under Olympus light microscope. Cells collection was done on day 12 by filtration through bolting cloth and subsequent centrifugation at 5000 rpm for 5-6 minutes. Supernatant was discarded and cells were used for RNA extraction.

Selection of culture conditions: Nutritional conditions were set according to Redfield ratio (Redfield, 1958), using four different concentrations of both fixed and independent N:P. For fixed N:P (16:1), high (HNP), and low nitrogen phosphorous (LNP) conditions were selected, while for independent N:P, both low nitrogen (LN) and high phosphorous (HP) culture media was selected. Medium nitrogen and phosphorous (MNP) was used as control (Table 1).

RNA isolation and reverse transcription for RT-qPCR: Trizol reagent (RNAiso Plus Takara Bio Inc. (Cat# 9108)) was used to isolate RNA. DNA was removed using Takara DNA-freeTM kit (Cat#1906). Using IMPLEN Nanophotometer (Pearl Nanophotometer GmbH, Germany) the concentration of each RNA sample was determined. RNA samples with 260/280 ratio between 1.9-2.1 and 260/230 ratio greater than 2.0 were selected for further study. The first-strand of cDNA was synthesized with total RNA (1µg) in 20 µl reaction using a *Clonetech Takara* Prime Script™ RT reagent Kit Japan (Cat.#RR047Q) according to the manufacturers protocol.

Primer design: All the primers utilized in this study were designed by Primer Premier 5.0. Primer sequences are listed in (Table) 2.

Cloning of ubiquitin gene from *A. catenella*: Based on the nucleotide sequence of transcriptomic *de novo* assembly (Zhang *et al.*, 2014), a primer pair UBF and UBR was designed for a unigene annotated to be ubiquitin to amplify the partial fragment. The PCR cycling parameters were set as 94°C for ten minutes, then 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min then 1 cycle of 72°C for 10 minutes.

Interestingly, a short sequence of 12 nucleotides was observed at the 5' end which was the exact match to part of Dino SL (Ou *et al.*, 2008). So Dino SL primer was paired with another reverse primer UBdino-R to obtain 340 base pair fragment using the above PCR protocol. PCR products were examined for size on 1% agarose gel and was then purified using TIANGEN Gel Purification kit (TIANGEN BIOTECH (BEIJING) CO. LTD.) as per manufacturer's instructions. Positive clones were sequenced to confirm the presence of desired fragment.

Sequence analysis: After assembling the initial partial PCR product and fragment obtained by Dino-SL primer using Mega 6.0, the encoded amino acid sequences were deduced from cDNA sequences using Unipro UGENE software. The homology of deduced amino acid sequence was investigated using Blastp. NCBI conserved domain database (CDD) search service was used to foresee the domain organization of deduced protein. The nucleotide sequence was also used as a query on local alignment search tool program Blastx against GenBank database. Key word "ubiquitin" was also used to search against protein database to pick any reported sequences which were not hit by Blastx search.

MUSCLE (Edgar, 2004), a tool freely available at the online server of the European Bioinformatics Institute (EMBL-EBI) was used to carry out multiple sequence alignment and JalView V 118 (Clamp *et al.*, 2004) was used to visualize the results. To examine the relationship between ubiquitin gene with counterparts from other organisms, deduced amino acid sequences of the *A. catenella* were aligned with dataset of respective sequences from the representative organisms. To construct a phylogenetic tree, statistical method described as Maximum Likelihood in Mega 6.0 was used. Poisson model was adopted for analysis and branch support was assessed through nonparametric bootstrapping using 1000 bootstraps. All other parameters were in default settings (Tamura *et al.*, 2013).

Table 1. Different kinds of media concentration used for the growth of *A. catenella.*

Ratio of phosphorous and nitrogen source	Symbol	N/P Ratio	Concentration of N µM/L	Concentration of P µM/L
Fixed N:P ratio	MNP	16:1	768	48
	HNP	16:1	2304	144
	LNP	16:1	76.8	4.8
Independent N:P ratio	LN	1.6:1	76.8	48
	HP	5:1	768	144

Table 2. Gene abbreviation, primer sequences, application and the length of amplicon.

Gene abbreviation	Forward and reverse primer sequence (5′-3′)	Application	Amplicon length
UBF	CCTGCCGCGCGTATAGAGCCAT	For ubiquitin gene	342 bp
UBR	TGCCCTCAGCCGTCTGCACACA	For ubiquitin gene	
DinoSL	CCGTAGCCATTTTGGCTCAAG	For Ub 5' amplification	340 bp
UbdinoR	CACATCAATCAGGTCCTCGTCC	For Ub 5' amplification	
qUBF	CTTGGGCTTGCAGGCTTCACAG	For qRT-PCR ubiquitin gene	112 bp
qUBR	GGCCACATCAATCAGGTCCTCGTC	For qRT-PCR ubiquitin gene	
Cob F	TTTTCTTCAAACTCATTTCGGGAT	Reference gene for qPCR	140 bp
Cob R	TGGGCACAGCTTTTAACATAGCATA	Reference gene for qPCR	
18S F	GGGGAGTATGGTCGCAAGGCTGA	Reference gene for qPCR	196 bp
18S R	CAAATCACTCCACCAACTAAGAACGG	Reference gene for qPCR	

To scrutinize the nucleotide sequences and characterize its deduced proteins, a series of online programs were utilized. GC content of the sequence was calculated using GC content calculator at www.endmemo.com/bio/gc.php. Physiochemical properties of the proteins were determined using ProtParam at ExPASy Bioinformatics Resource portal available at http://web.expasy.org/protparam. To foresee conserve domains of the proteins, first NCBI conserved domain database was used and then it was cross checked at Pfam (pfam.xfam.org) and InterProScan sequence search (www.ebi.ac.uk/interpro).

Quantitative RT-PCR analysis: The qRT-PCR reaction was carried out in a Multiwall (96-wall) plate in triplicates for both technical and biological replicates in Applied Biosystems 7500 Real-Time PCR System using SYBR Green Real time PCR Master Mix (Applied Biosystems Ltd.). The qPCR reaction mixture consisted of SYBR green (10 µl), 10 mM specific forward primer (0.6 µl), 10 mM reverse primer (0.6 µl), RNase-free H_2O (6.8 µl), and 2 µl cDNA. To optimize the PCR conditions and to ensure that every primer yields single amplification product, the amount of primers and cDNA was adjusted. Single amplification product was evident from single peak in dissociation curve. Primers having single peak were selected for further experiments. Each plasmid having target fragment was diluted in steps of 10-fold till eight tubes. Each dilution was used as a template in triplicates to determine amplification efficiency and standard curve. By using at least 5 consecutive dilution points measured in triplicates, a standard amplification curve was constructed for each gene. A no-template negative control using sterilized water was also included in each PCR. The program of RT-PCR was 2 min at 50°C, 5 min at 95°C followed by 40 repeats of 15 sec at 95°C, 1 min at 60°C, one cycle of 15 sec at 95°C, 1 min at 60°C, one cycle of 15 sec at 95°C, 15 sec at 60°C. Cob and 18s were used as reference genes for internal control in this experiment, which had been broadly used in various marine algae including those of *Alexandrium*. The primers of real time quantification for ubiquitin, Cob and 18S are listed in Table 1. To assess the gene expression of target genes under different nutritional conditions listed in table 1, $2^{-\Delta\Delta CT}$ method was used to evaluate the fold change (Livak & Schmittgen, 2001) while cDNA from cells grown under medium nitrogen and phosphorous (MNP) was used as endogenous positive control.

Results

Sequence analysis of ubiquitin gene: A partial fragment of 342 bp (Fig. 1) from 474 bp unigene annotated to be ubiquitin in the transcriptome sequence data of *A. catenella* was amplified. Interestingly at 5' UTR of transcriptome sequence, a 12 bp short region matching to dinoflagellate spliced leader was observed and was amplified using forward Dino SL primer reported earlier (Ou *et al.*, 2008) and UBR-SL primer. A fragment of 340 bp (Fig. 1) was obtained. Assembly of both the sequences of PCR products yielded a fragment of 407 bp (Fig. 2).

Analysis of the nucleotide sequence by Blastx revealed that the amplified sequence possesses high similarity with small ubiquitin-like protein of *Pfiesteria piscicida* (Fig. 3), therefore we concluded that the cloned sequence was that of ubiquitin gene in *A. catenella.* Analysis of the amplification product of 407 nucleotides of ubiquitin in *A. catenella* identified a complete open reading frame containing (ORF) of 285 nucleotides with a GC content of 63.85% encoding 94 amino acid residues. The calculated molecular weight was about 10.06 kD with estimated pI (isoelectric point) of 4.64. The amino acid sequence contains 17 negatively (Asp+Glu) and 11 positively (Arg+Lys) charged residues. On the whole, the percentage of alanine and glycine amino acids was the highest with 11.7% and 10.60% respectively. The instability index was calculated to be 24.99 which classify the protein as stable.

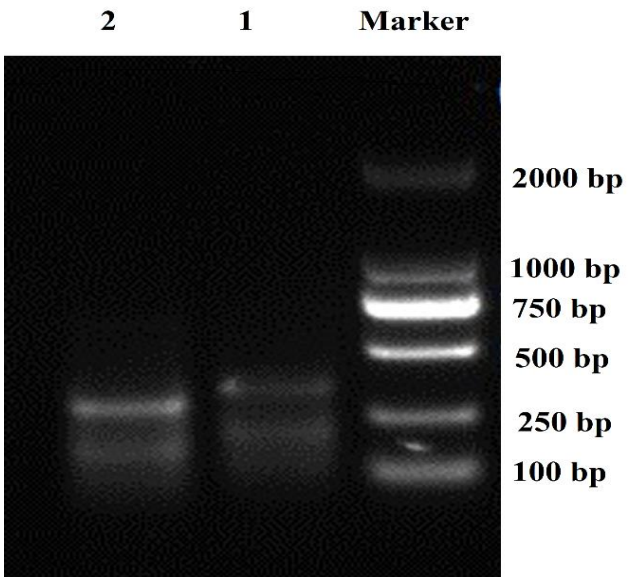

Fig. 1. Amplification of ubiquitin gene. 1 represents the partial amplification product of 342 bp using UBF and UBR primer pair while 2 represents the 340 bp fragment amplified using Dino-SL and UbdinoR primer pair.

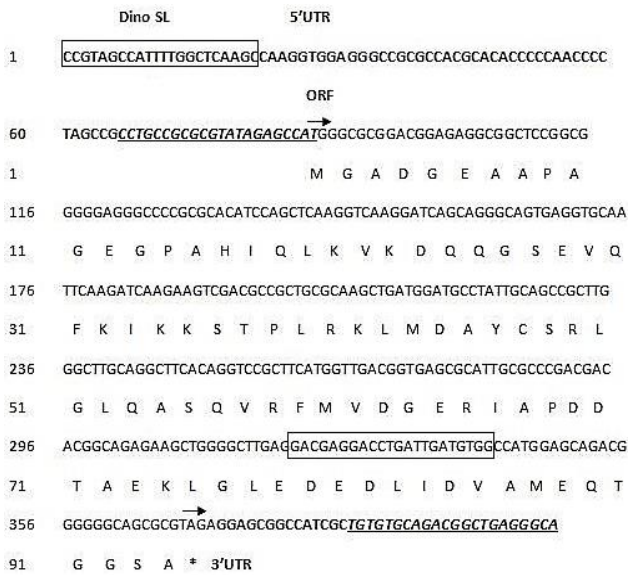

Fig. 2. Sequences of cDNA and deduced amino acid sequence of Ubiquitin in *A. catenella*. Boxed regions at the start is DinoSL sequence present at the 5' end, while the second boxed region is "UBdino-R" is a reverse primer used in combination with Dino SL. Italicized and underlined part of the sequence are forward and reverse primers for primary amplification product. Initiation codon "ATG", and stop codon "TAG" are represented by forward arrow on the top, Bold sequences at each end represents 5' and 3' UTR's respectively.

Examination of the amino acid sequence by Blastp and conserved domain database revealed that the cloned sequence belongs to superfamily of ubiquitin and list of domain hits included small ubiquitin related modifier (6-92) and ubiquitin homologous region (17-88) involved in ubiquitin mediated proteolysis (Fig. 3). The cloned sequence contained the conserved domains of ubiquitin including the hydrophobic surface conserved amino acid regions and Ulp1-Smt3 interaction sites. Alignment of the cloned sequence further shown that the types of amino acid sequence are different in top 5 and bottom 5 sequences obtained from GenBank, however both same and different polar types represent the same conserved region as observed at Conserved domain database (CDD). For example, the hydrophobic surface conserved amino acids are shown in figure 3 box 1 to box 5 (GE, Q, K, K and R). The Glycine (G) and glutamic acid (E) hydrophobic amino acids are conserved among *A. catenella* and *P. piscicida,* while in other species glycine is either replaced by glutamine (Q), an another non polar and neutral amino acid (M), while glutamic acid (E) conserved amino acid having acidic polarity with negative side chains is replaced by basic polar lysine (K) as shown in figure 3 box 1. Glutamine (Q) in box 2 is replaced by asparagine (N) in some species both representing the same class of polar, neutral amino acids. Similarly, in 4th, 5th and 6th hydrophobic region (box 3 and 4 and 5 respectively in figure 3) lysine (K) is replaced by arginine (R) both basic polar amino acids with positive side chains.

These hydrophobic regions are thought to interact with modifying/demodifying enzymes (Bayer *et al.*, 1998) and twelve Ulp1-Smt3 interaction sites (R, VDG, R, A, D, G, D and AME) where Ulp1 binds Smt3. The first amino acid arginine (R) representing Ulp1-Smt3 interaction site is highly conserved among the species (box 6 figure 3). The 2nd and 3rd Ulp1-Smt3 interaction sites were found similar between *A. catenella* and *P. piscicida,* while in rest of the species, non-polar valine is replaced by either phenylalanine (F) which belongs to their class of amino acids or by polar neutral tyrosine (Y) (box7 figure 3). On the other hand, the 4th Ulp1-Smt3 interaction site represented by glycine (G) is highly conserved across the species. Similarly, arginine (R), the 5th Ulp1-Smt3 interaction site is conserved among half of the species while in others it is replaced by glutamine (Q) which belongs to different class of amino acids (box 8 figure 3.3). At 6th Ulp1-Smt3 interaction site alanine (A) is replaced by asparagine (N), serine (S) and glutamic acid (E) representing different class of amino acids. At 7th Ulp1-Smt3 interaction site, aspartic acid (D) is common among three species while in the others it is replaced by asparagine (N), glutamine (Q), alanine (A) and arginine (R) that represents different class of amino acids (box 10 figure 3). The 8th Ulp1-Smt3 interaction site glycine (G) in *A. catenella* is conserved among four species while in the others it is replaced by aspartic acid (D) and asparagine (N). The 9th Ulp1-Smt3 interaction site aspartic acid (D) is conserved among five analyzed sequences while in the others it is represented by phenylalanine (F) and histidine (H) that belong to different class of amino acids. The 10th, 11th and 12th Ulp1-Smt3 interaction sites containing alanine (A), methionine (M) and glutamic acid (E) are conserved in *A. catenella* and *P. piscicida* while in the others they are replaced by variety of different amino acids belonging to both same and different classes as shown in box 1-13 figure 3. Phylogenetic tree constructed from the deduced amino acid sequences revealed two distinct clades and both the clades have ubiquitin sequences from dinoflagellates. In one clade, ubiquitin genes of dinoflagellates were clustered together with algal species from chlorophyta and rhodophyta. In another clade, three sub-clades were observed where the deduced amino acid sequence of ubiquitin that belongs to *A. catenella* was clustered with another dinoflagellate *P. piscicida* with 100% bootstrap value (Fig. 4).

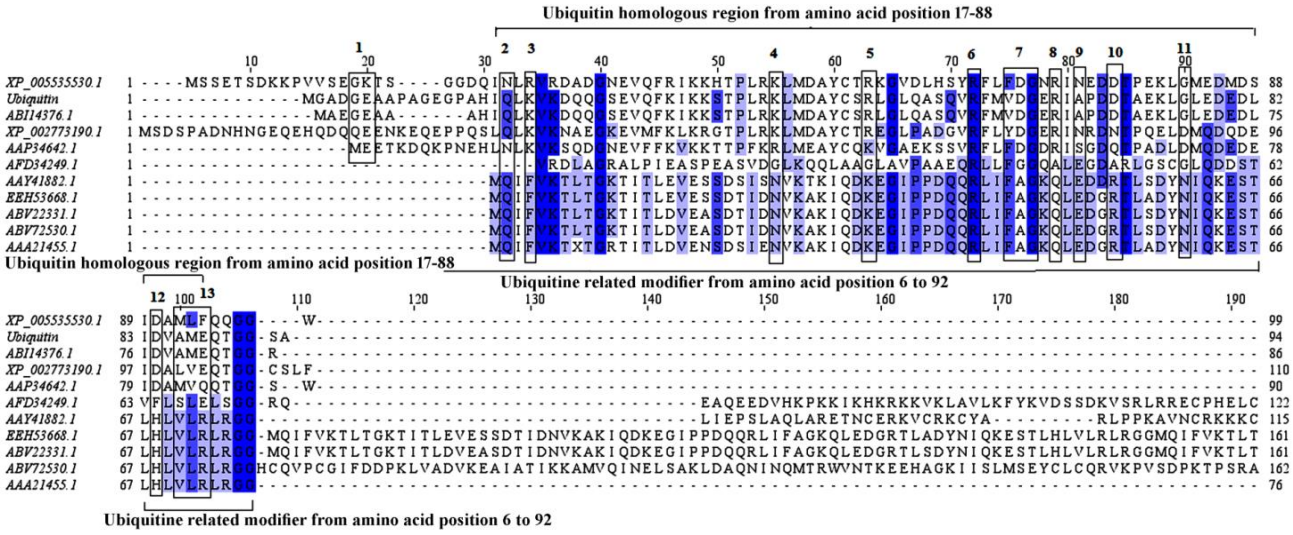

Fig. 3. Homology alignment and structure domain analysis of ubiquitin amino acid sequences of *Alexandrium catenella*. Box 1 to 13 represents the conserved hydrophobic surface regions and conserved Ulp1-Smt3 (ubiquitin like protein-SUMO protein) interaction sites of ubiquitin. Ubiquitin indicates the amino acid sequence of ubiquitin of *A. catenella*. Sequences taken from GenBank were: *Cyanidioschyzon merolae* strain (XP_005535530.1), *Perkinsus marinus* (XP_002773190.1), *Bigelowiella natans* (AAP34642.1), *Pfiesteria piscicida* (ABI14376.1), *Prorocentrum minimum* (AFD34249.1), *Gracilariopsis lemaneiformis* (AAY41882.1), *Micromonas pusilla* (EEH53668.1), *Noctiluca scintillans* (ABV22331.1), *Heterocapsa triquetra* (ABV72530.1) and *Symbiodinium spp*. (AAA21455.1). Phylogenetic tree constructed from the deduced amino acid sequences revealed two distinct clades and both the clades have ubiquitin sequences from dinoflagellates as shown in fig. 4. In one clade, ubiquitin genes of dinoflagellates were clustered together with algal species from chlorophyta and rhodophyta. In another clade, three sub-clades were observed. Ubiquitin of chlorarachiniophyte alga, *Bigelowiella natans* forms a sub clade with a protist *Perkinsus marinus* that is believed to be similar to dinoflagellates (Saldarriaga *et al.*, 2003; Joseph *et al.*, 2010). This extends to make another sub clade where the deduced amino acid sequence of ubiquitin that belong to *A. catenella* was clustered with another dinoflagellate *P. piscicida* with 100 % bootstrap value.

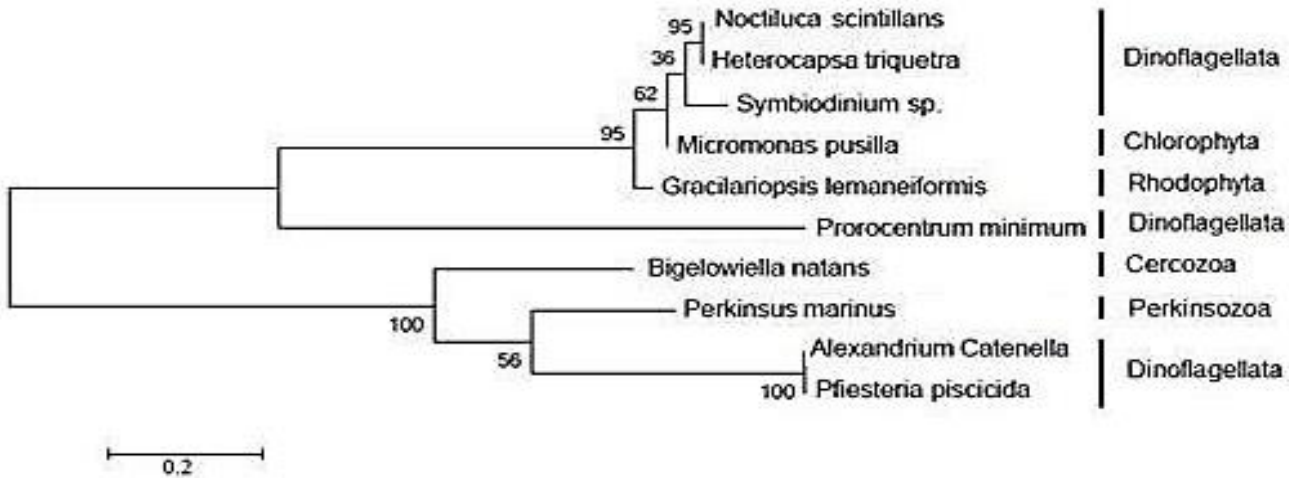

Fig. 4. Phylogenetic analysis of Ubiquitin amino acid sequences. Number at the end represents the bootstrap values. Scale indicates the evolutionary distance among the species. Accession numbers of the gene sequences of the respective organisms are given in figure legend 3.

Quantitative RT-PCR analysis: RNA samples extracted from *A. catenella* all displayed good quality, with A260/A280 ratios ranging from 1.97 to 2.14, and A260/A230 ranging from 1.98 to 2.82. The appearance of single band with predictable size on gel, single peak of dissociation curve after RT-qPCR and sequencing results established the specificity of the primers. Absence of product in the negative control confirms the absence of genomic DNA contamination thus indicating the reliability of RT-qPCR results. The correlation coefficient (R^2 value) for both target (ubiquitin) and control genes (Cob and 18S) were well within the normal limits. The amplification efficiency was also recorded to be between 95.33 % to 99.08% for all the four genes which is within the standard limits (Fig. 5).

The analysis by $2^{-\Delta\Delta ct}$ method demonstrated that both ubiquitin and polyubiquitin genes were upregulated under all the experimental conditions. Fold change for ubiquitin gene was 9.75, 219, 47 and 3.26 at HNP, LNP, HP and LN respectively (Fig. 6). Although the genes are upregulated at all the conditions but the fold change was very high at LNP.

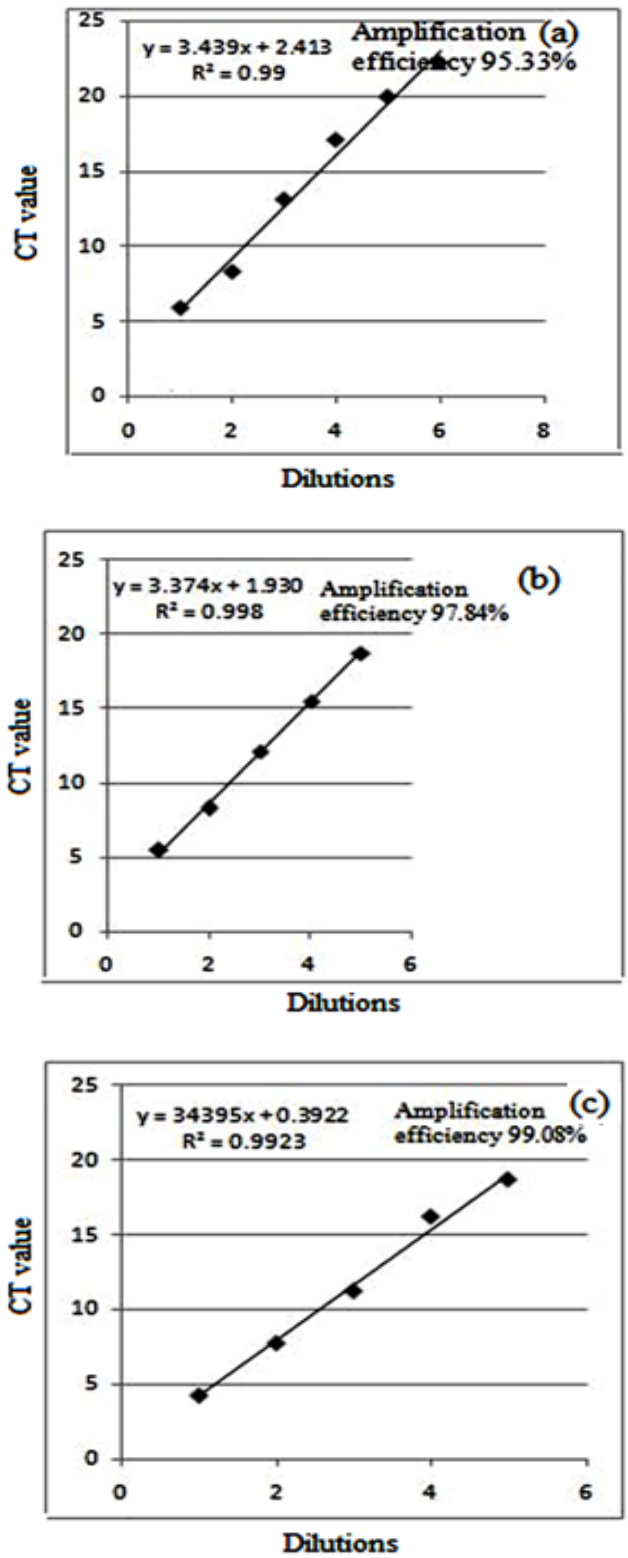

Fig. 5. a-c- shows the standard curves of the target gene (ubiquitin) and reference genes (*18S* and *Cob*).

Discussion

Regulated synthesis of new polypeptides and degradation of defined preexisting proteins control every feature of plant life. In plants approximately fifty percent of the total cellular proteins are replaced through this protein cycle (Vierstra, 1993). This protein cycle (proteolysis) play a vital housekeeping role by eradicating abnormal or misfolded proteins (De Strooper, 2010), and providing free supply of amino acids during growth and nutrient scarcity (Silk, 1974; Vierstra, 1996). It is crucial for many aspects of cellular regulation e.g., remove rate-limiting enzymes and dismantle existing regulatory networks to maintain homeostasis, adaptability to new environment and redirecting cellular growth and development (Vierstra, 1996; Hellmann & Estelle, 2002; Vierstra, 2003).

Extreme environments negatively influence proteins by escalating free radicals that support denaturation and damage. Eradicating these proteins by a range of quality control pathways within the ubiquitin proteasome system is critical for cell survival (Kopito, 2000; Kostova & Wolf, 2003; Varshavsky, 2003). Lysosome-mediated intracellular protein degradation and the proteasome-mediated protein degradation are the two important mechanism of cellular proteolysis that plays a key role in cellular homeostasis. Ubiquitin is virtually ubiquitous; being present in all eukaryotic species examined and is highly conserved. Its amino acid sequence is invariant amongst higher plants and it differs from yeast and animal ubiquitin by 2 and 3 residues respectively (Callis & Vierstra, 1989).

In the deduced amino acid sequence of ubiquitin of *A. catenella*, hydrophobic surface conserved regions are present at N-terminus region while the majority of ubiquitin essential residues i.e. the protein interaction sites map to the C-terminal tail. An interacting site is extremely conserved (Keskin *et al.*, 2004; Keskin & Nussinov, 2005) and co-evolved (Pazos *et al.*, 1997) short sequence of residues which form the contact interfaces between two interacting proteins and mediate protein interaction (Sheu *et al.*, 2005; H. Li *et al.*, 2006). These interactions are vital for almost all aspects of cells including immunological recognition, gene regulation and proteins synthesis etc. (Zhou, 2004; Zhou & Qin, 2007). The bulk of key hydrophobic surface ubiquitin residues mapped to the N-terminus of ubiquitin in *A. catenella* are thought to be associated with modifying and demodifying enzymes (Bayer *et al.*, 1998).

Twelve Ulp1 - Smt3 interaction sites on the conserved domain were found at the C-terminus of the deduced protein as reported earlier (Johnson, 2004). Ubiquitin like SUMO protein also known as “Smt3” or “sentrin” in other organisms play vital role in many cellular regulatory mechanisms via protein modification. Sumoylating and desumoylating activities are believed to have essential role in cellular viability in *Saccharomyces cerevisiae.* Ubiquitin like specific protease (Ulp1) is well known desumoylating enzyme explicitly requisite for cell cycle progression (Li & Hochstrasser, 2003). In SUMO pathway Ulp1 protease catalyzes the processing of full-length of Smt3 to its mature form and its deconjugatation from target proteins. Covalent thiohemiacetal transition state complex between Ulp-Smt3 is produced as a result of selective reduction of the proteolytic reaction. The Ulp1-Smt3 within the conserved interface illuminate the determinants of SUMO recognition, processing and de-conjugation (Mossessova & Lima, 2000).

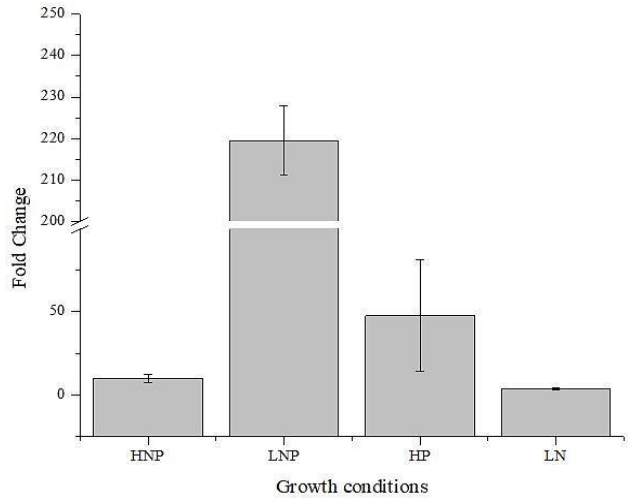

Fig. 6. Represents fold change obtained through quantitative RT-PCR for ubiquitin (UB) gene of *A. catenella* grown under different nutritional conditions (HNP, LNP, HP and LN). Each bar in the graph represents the mean of three determinations on a sample with standard deviation.

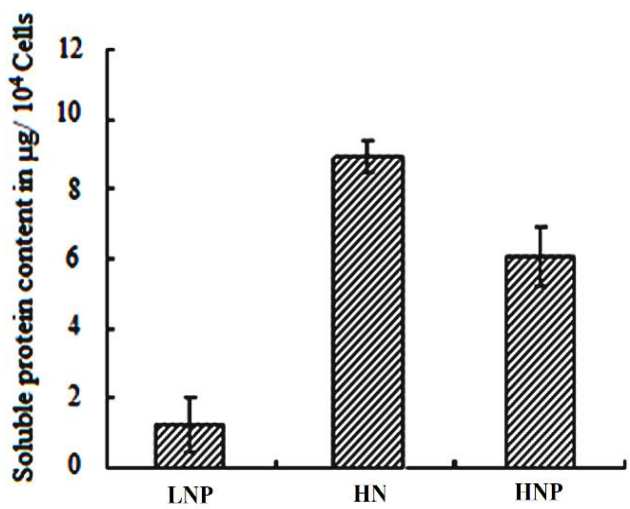

Fig. 7. Change of soluble protein content in *A. catenella* under different N/P level (Ren, 2015).

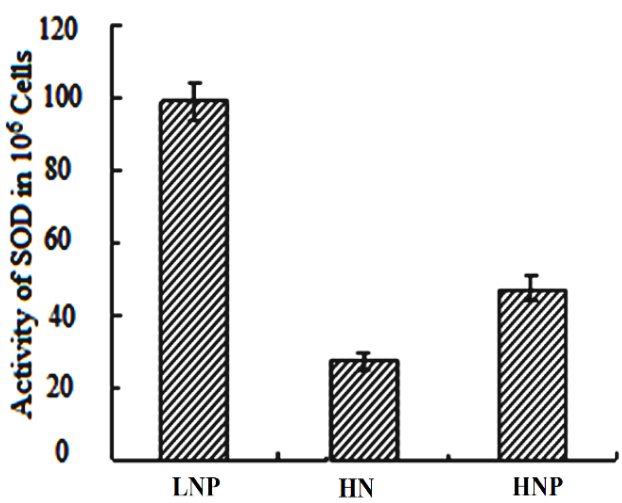

Fig. 8. Change of SOD activity in *A. catenella* under different N/P level in culture grown for 12 days (Ren, 2015).

The expression analysis by $2^{-\Delta\Delta ct}$ method using RT-qPCR showed that ubiquitin gene was significantly upregulated under all experimental conditions. Aging is a natural process of dying plants that is affected by external factors controlled by genes (Xianyun, 2006). Physiological and biochemical changes occur during the process of aging (Xianhua, 2006): The first of among these changes is protein degradation. Study on the effects of cadmium stress on the growth and antioxidant system of *Isochrysis* and *Chlorella vulgaris* indicated that high concentrations of cadmium in two algae results in growth inhibition and decrease in soluble protein content (Tian Dan, 2010).

Inside the algal cell, the soluble protein content displays the growth state of the cells. Previous studies in *A. catenella* reported that under sufficient NP concentrations in the medium, physiological state of the algal cells are better those results in higher soluble protein content. On the other hand, lower concentration of nutrients in the medium results in decreased cellular metabolism, reduced protein synthesis and reduced soluble protein content in the cell (Ren, 2015). Around 30% of early translation products are nonfunctional and are removed by Ubiquitin/26S proteasome pathway (Schubert *et al.*, 2000). These reports advocate the fold change observed in our study where both ubiquitin and polyubiquitin are found upregulated at HNP and HP condition.

In the decline phase of *A. catenella*, significantly lower expression of ubiquitin gene was reported that show the decline of protein degradation UPP pathway and reduction of soluble protein content (Ma, 2013). However, in the earlier report of *A. catenella* (Ren, 2015) where soluble protein content was found in low concentration at day 12 under LNP condition as shown in figure 7, the cell count was similar to the one obtained in this study and the algae was found to grow at low rate and enter decline phase after day 13. It supports that upregulation of ubiquitin at LNP condition in our study.

Fold change recorded for ubiquitin at LN was 3.62, which showed that ubiquitin gene is upregulated. In nitrogen-limited environments, plants are reported to usually degrade proteins in old mature leaves that serve as main source of nitrogen, and export them to actively growing tissues such as young leaves, flowers and seeds to maintain essential cellular functions. Along with autophagy (Gönenç *et al*., 2020), active participation of ubiquitin. proteasome is reported in this process (Smalle & Vierstra, 2004; Thompson & Vierstra, 2005). In *Arabidopsis thaliana*, 21genes functioning in autophagy and ubiquitin proteasome pathways are reported to be upregulated in response to nitrogen limitation, that includes seven genes encoding various ubiquitin ligases (2 to 3 fold upregulation) (Peng *et al.*, 2007). In *nla* (nitrogen limitation adaptation) mutant of *Arabidopsis,* the number of encoded genes in response to nitrogen limitation further increases. In ubiquitination-mediated protein degradation, the seven genes exclusively code for ubiquitin ligases while in contrast in *nla* mutant also encode ubiquitin, polyubiquitin, ubiquitin-conjugating enzyme, ubiquitin transferase, and subunits of 20S and 26S proteasomes (Peng *et al.*, 2007). After day 10, dead

cellular debris began to appear in the LN medium but at the same time algae was found to grow slowly and reached at its highest on day 13 after which decline was observed. It is presumed that algae were growing at the expanse of dead cellular debris.

Similar phenomenon was observed in *Arabidopsis* where both wild type and *nla* mutant were found to activate genes involved in protein degradation to get hold of nitrogen and endure the nitrogen limited growth conditions. More interestingly, none of the gene involved in protein synthesis was up regulated by nitrogen limitation in wild type specie (Peng *et al.*, 2007). Most of the cellular proteins found in prokaryotes and eukaryotes are stable *in vivo* (Mosteller *et al.*, 1980) but upon exposure to stress or abnormal environments stable proteins could become unstable (Thiel, 1990). Some rapidly degrading proteins perform vital regulatory roles in metabolic and developmental process (Mosteller *et al.*, 1980; Thiel, 1990; Hörtensteiner & Feller, 2002). For example, in *Alexandrium affine*, Rubisco II and NAP-50 proteins were found stable at nitrogen replete condition and for short time in nitrogen depletion but there levels were found to be drastically decreased between 30 to 48 hours in nitrogen depleted conditions that show rapid and regulated degradation of the two proteins. However mRNA levels of both the proteins are found constant and independent of the condition that excludes transcriptional control as possible mechanism for their regulation (Lee *et al.*, 2009). Every protein found in living organism have different and characteristic lifetime that is why turnover rates and quality control of proteins are physiologically vital for the organism (Gottesman *et al.*, 1997) and can be affected by environmental condition or developmental state, e.g. in plants, protein degradation occurs during senescence (Hörtensteiner & Feller, 2002) which shows that it is a complex and highly regulated developmental phase. The degradation of proteins like rubisco provides amino acids that are transported to other parts of the plant.

After subjecting cells of *A. catenella* to LNP condition, slow growth was observed but the algae continue to grow until day 13 even after phosphate depletion. The fold change observed for both ubiquitin and polyubiquitin genes at LNP were found to be significantly higher as compared to another report in *A. catenella* (Ma, 2013). The possible reason might be that the previous study reported the down regulation of ubiquitin in decline phase where the alga was initially provided with sufficient nutrients. On the other hand, we report the up regulation of both ubiquitin and polyubiquitin genes in algae grown under LNP medium where the *A. catenella* was still growing at slow rate on day 12 and decline was observed after day 13.

Oxidative stress due to excessive reactive oxygen species (ROS) and its derivatives can damage cells and almost all of its components including carbohydrates, proteins, DNA and lipids that lead to cellular dysfunction. In response to ROS, organisms have developed a variety of defense mechanisms including antioxidants and antioxidant enzyme activity, molecular chaperone systems and protein degradation in order to reduce oxidative damage. Selective degradation of oxidative damage of proteins have been found in different types of cell or tissue (Grune *et al.*, 1997; Gieche *et al.*, 2001; Balog *et al.*, 2009). Ubiquitination and proteasomal degradation of substrates of ubiquitin proteasome system have been associated to ROS as important components of UPS counting proteasome and every class of enzyme implicated in ubiquitination are sensitive to redox (Kriegenburg *et al.*, 2011). The enzymatic activities of the ubiquitin-conjugation system are enhanced in reaction to a range of ROS inducers resulting in increased number of Ub-conjugates (Gomes-Marcondes & Tisdale, 2002; Shang & Taylor, 2011). These result advocates the positive impact of ROS on cellular UPS function. Similar results were found in a study conducted on tobacco where regulation of ubiquitin precursor proteins, ubiquitin-conjugating enzymes (E2), and ubiquitin-protein ligases (E3) is triggered by hydrogen peroxide (H_2O_2) (Vandenabeele *et al.*, 2003).

Preceding work conducted on *A. catenella* subjected algae to the fixed and independent concentration of nitrogen and phosphorous for 12 days and determined the activity of superoxide dismutase (SOD). At higher concentration of nutrients, growth rate was high while SOD activity was low. While high activity of SOD was observed in the group provided with low concentration of nutrients as shown in figure 8 (Ren, 2015). Due to exhaustion of nutrients in the group of LNP and LP (Low phosphorous), large amount of ROS are produced and in response SOD are actively generated in the algal cells for their protection (Ren, 2015). Comparison of gene expression pattern obtained in our study with the results of (Ren, 2015) shows that our results are strongly in accord with earlier reports.

Even though, the relationship between ROS and UPS activity is strongly supported by many studies, impaired UPS function has also been linked with ROS (Huang *et al.*, 2013; Segref *et al.*, 2014). Although these results are not entirely clear, these conflicting outcomes may be somewhat endorsed to the presence of diverse proteasome populations and the disparities in experimental conditions.

The results presented here shows the up regulation of ubiquitin and polyubiquitin gene when algae were grown under limited phosphorous and nitrogen condition, where the algae were growing slowly while the preceding reports only either focused on their expression either at low nitrogen, low phosphorous or at the decline phase.

Conclusion

Currently little is known about protein degradation under different nutritional condition in microalgae, especially in dinoflagellates and the precise function and mechanisms of protein degradation through ubiquitin-proteasome needed to be addressed in future studies. This study provides an important first look into the relationship between nutrient supply and expression analysis of ubiquitin in dinoflagellates.

In the light of above results it can be concluded that ubiquitin genes is upregulated at all the conditions except at LN condition. which included log phase of *Alexandrium* at eutrophication and slow growth at

nutrient limited conditions such as LNP and LN, so ubiquitin protein play an important role when algae are rapidly growing and excess proteins are degraded by ubiquitin proteasome system. However in nutrient depleted conditions where the algae grow slowly and culture is under the effects of ROS (Ren, 2015), high activity of SOD enzyme triggers the activation of UPS, as a result ubiquitin gene was highly upregulated as compared to other conditions.

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