EXTRACELLULAR PROTEASE PRODUCTION BY WATERMOULDS DETERMINES THEIR VIRULENCE DURING PATHOGENESIS ON FISH

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ABSTRACT: An attempt has been made to assess the qualitative and quantitative production of protease enzyme by pathogenic watermoulds, viz., *Saprolegnia diclina*, *Aphanomyces laevis*, *Achlya diffusa*, *Achlya proliferoides* and *Pythium sp.* and to determine any correlation between severity of infection and the protease production. The observed data showed that there was maximum protease production by each test fungi at pH 7.5 and at 25ºC, while the slightly acidic pH 6.5 showed the minimum extracellular protease production. At 72 h incubation period maximum extracellular protease production has been observed by all the test fungi at 25ºC and 7.5 pH range. The present results distinctly indicate towards a direct correlation between protease production by the water moulds and their virulence during their pathogenesis on fishes.

KEYWORDS: Extracellular protease, Water moulds, Virulence, Fish, Pathogenesis

INTRODUCTION

Fish is one of the man's oldest foods, and because of its importance as a rich source of protein, it is of great nutritional value. In view of the rapid growth of the world's population, it seems advisable to be economical with this important source of very rich, palatable and easily digested protein. Fungi, particularly watermoulds, are known to attack eggs, fry, fingerlings and adults of fishes and as a general rule the fungal infection starts when the host gets injured either mechanically or as a result of infections other than fungal. These watermould pathogens make the infected fishes lethargic and ultimately cause mortality resulting into colossal loss to fish industry.

Earlier observations[1] indicate that there is significant depletion of muscle protein content (Fig. 1) due to fungal infection which is indicative of protein biodegradation caused by proteolytic enzyme (protease) produced by the pathogenic watermoulds. Srivastava and Prabhuji[2] have studied

the effects of variation in nutrients on growth and extracellular protease production in *Trichophyton rubrum*, a human dermatophyte; and found that when easily metabolized substrates aremade available to *T. rubrum*, the protease activity is not induced; in contrast nutrient depletion or growth as stationary phase cultures increases the production of protease. The previous literatures have indicated that no work has yet been done on the extracellular production of protease in pathogenic watermoulds and its significance on their virulence during pathogenesis on fish.

MATERIALS AND METHODS

Qualitative analysis

For qualitative screening of protease production by watermoulds the fungal isolates were inoculated on the medium (Table 1) and incubated at 25˚C for 48 hours; and then, the plates were observed for "zones of lysis" developed due to protease production (Figs. 2 A, B, C, D).

Quantitative analysis

The quantitative protease assay was performed[3] with a few modifications. To 1.0 ml of 1.0 % (w/v) substrate (casein solution) in 0.1M phosphate–phosphate buffer pH (6.5, 7.5 and 8.5) 0.2 ml of enzyme solution was added. The reaction mixture was kept at 30^{o}C for 1 hour after which the reaction was stopped by the addition of 20% TCA (Tri Chloro-Acetic acid) and after a lapse of 10 minutes time the reaction mixture was centrifuged at 10,000 rpm for 10 minutes. To 1.0 ml of the supernatant, 5.0 ml of 0.275 M sodium carbonate, 2ml of DW and 0.5 ml of 1:1 diluted Folin's phenol reagent were added. A blue colour developed in the test samples. The Optical Density (OD) was measured at 660 nm using Genitix Asia Biotech Spectrophotometer VIS-7220G.

OBSERVATIONS

Following experimentation, the protease production has been observed in watermoulds, viz., *Saprolegnia diclina*, *Aphanomyces laevis, Achlya diffusa, Achlya proliferoides* and *Pythium sp.* and its assessment has been made qualitatively as well as quantitatively. The *Pythium sp.* has been identified, later, as *Pythium oligandrum* and its fish tissue destructive nature has been described[4]. During quantitative assessment the effect of pH, temperature and period of incubation have been taken as parameters to assess the production of protease.

Qualitative assessment of protease

Qualitative screening of protease production by selected watermoulds, exhibited the variable production of extracellular protease on the test plates containing Skimmed milk Agar media

by variable formation of zones of inhibition after 48 h incubation. *Aphanomyces laevis* showed maximum zone of inhibition and *Achlya diffusa* showed the minimum whereas *Saprolegnia diclina* and *Pythium oligandrum* showed an intermediate state in between maximum and minimum (Figs. 2 A-D). The screening test provided a basic guideline to access

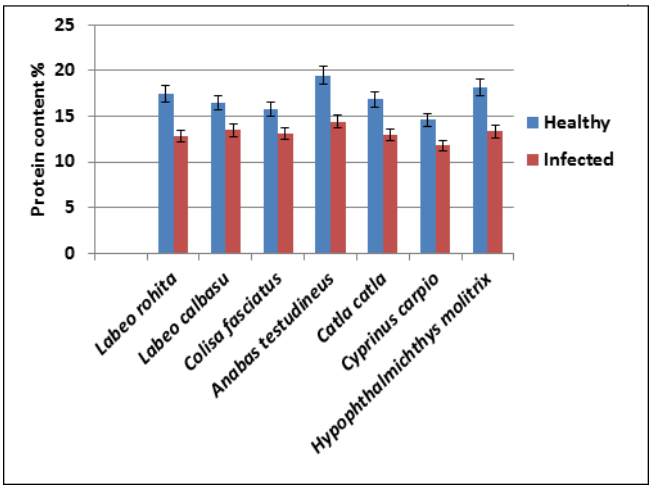

Fig.1: Variation in muscle protein content of different healthy and infected Fresh-water fishes (Ahmad *et al.* [1])

Table 1: Modified Skimmed milk agar medium[15]

1.	**Yeast Extract**	**4.0 g**
2.	**Beef Extract**	**3.0 g**
3.	**Skimmed milk powder**	**5.0 g**
4.	**Potassium Dihyrdogen Phosphate**	**1.0 g**
5.	**Potassium Monohydrogen Phosphate**	**1.0 g**
6.	**Dextrose**	**4.0 g**
7.	**Agar**	**12.0 g**
8.	**Distilled Water**	**1000 ml**
9.	**pH**	**7.5**

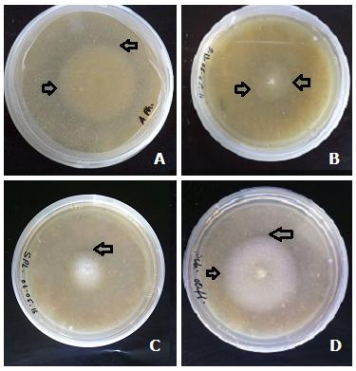

Fig. 2: Screening test for protease production in watermoulds (Arrows indicate the lytic zones)
A: *Aphanomyces laevis*; B: *Pythium oligandrum;*
C: *Saprolegnia diclina*; D: *Achlya diffusa.*

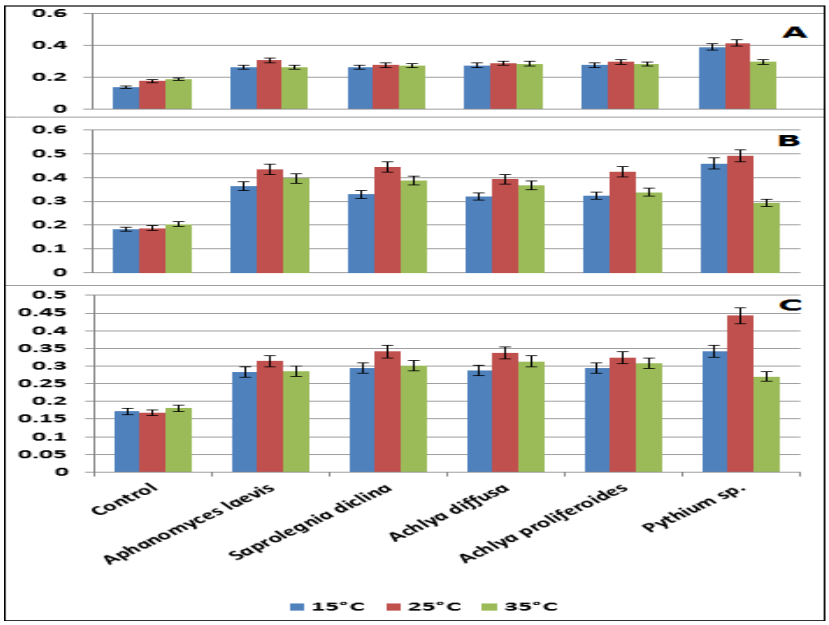

Fig. 3: Protease activity (specific activity µmol/mg-min) at different pH on 48 h incubation (A: pH 6.5; B: pH 7.5; C: pH 8.5)

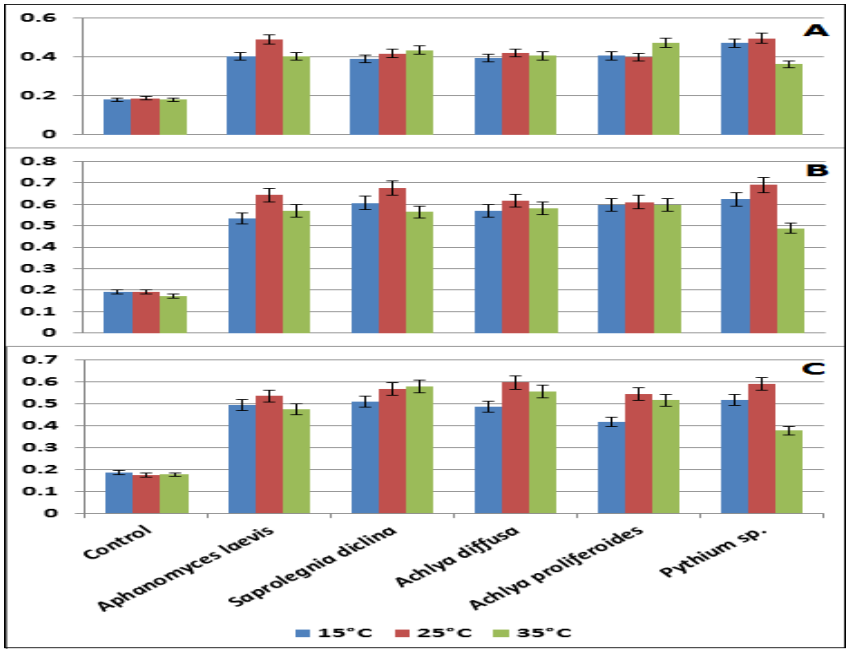

Fig. 4: Protease activity (specific activity µmol/mg-min) at different pH on 72 h incubation (A: pH 6.5; B: pH 7.5; C: pH 8.5)

extracellular protease production quantitatively.

Quantitativeassessment of protease

During quantitative assessment of extracellular protease production by different watermoulds the data indicated variation under different parameters, viz., pH, temperature and the period of incubation.

pH variation and production of protease:

pH is an important factor for the growth of the watermoulds. During the present investigation for quantitative assessment of extracellular protease different pH ranges have been selected viz., 6.5, 7.5 and 8.5 with selected temperature ranges which was constant for each pH range (Figs. 3-8). The observed data showed that there was maximum protease production by each test fungi at pH 7.5which was the best range at 25ºC, while the slightly acidic 6.5 showed the minimum extracellular protease production whereas the basic condition, i.e., 8.5 was intermediate at the same temperature.

Temperature variation and production of protease:

Low temperature range, less than 30ºC, has been found to be favourable for the growth of watermoulds. For the quantitative assessment of extracellular protease production different temperature ranges have been selected viz., 15ºC, 25ºC and 35ºC with selected

pH ranges which was constant for each temperature range (Figs. 3-8). The observed data showed that there was maximum protease production by each test fungi at 25ºC which was the best range at all pH ranges, while 35ºC showed the minimum extracellular protease production whereas the 15ºC temperature has shown an intermediate state.

Incubation period and production of protease:

At 72 h incubation period maximum extracellular protease production has been observed by all the test fungi at 7.5 pH and 25ºC, however, minimum production was at 96h incubation period which fluctuated near the range of control condition and the intermediate production level has been observed at 48 h incubation period (Figs. 3-8).

RESULTS AND DISCUSSION

The studies have indicated that the maximum protease production takes place at 25ºCtemperature with 7.5 pH range and after 72 h period of incubation. These conditions have been shown to be favourable for maximum growth, nutrient consumption and protease production by *Trichophyton rubrum*, a human dermatophyte[2]. It has also been reported that afore said physical conditions for the growth of these fungi is the best on the basis of dry weight obtained from cultured watermoulds[5]. On the basis of present investigations and earlier data it has been found that slightly basic pH range has been very favourable for protease production at temperatures ranging between 20 - 30ºC.

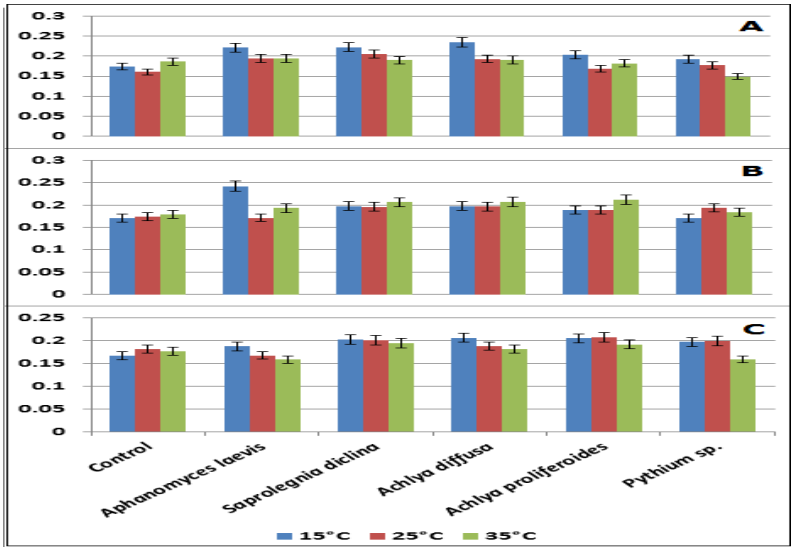

Fig. 5: Protease activity (specific activity µmol/mg-min) at different pH on 96 h incubation (A: pH 6.5; B: pH 7.5; C: pH 8.5)

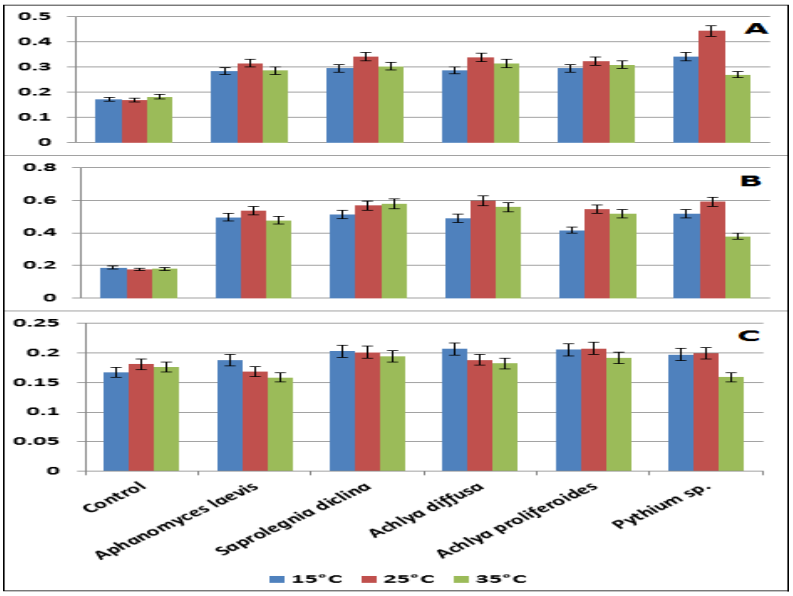

Fig. 6: Protease activity (specific activity µmol/mg-min) at pH 6.5 on different incubation period (A: 48 h; B: 72 h; C: 96 h)

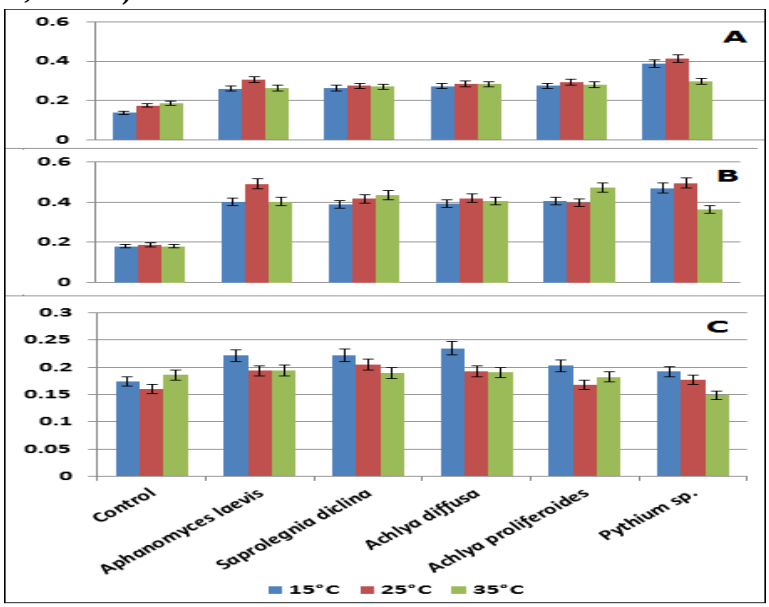

Fig. 7: Protease activity (specific activity µmol/mg-min) at pH 7.5 on different incubation period (A: 48 h; B: 72 h; C: 96 h)

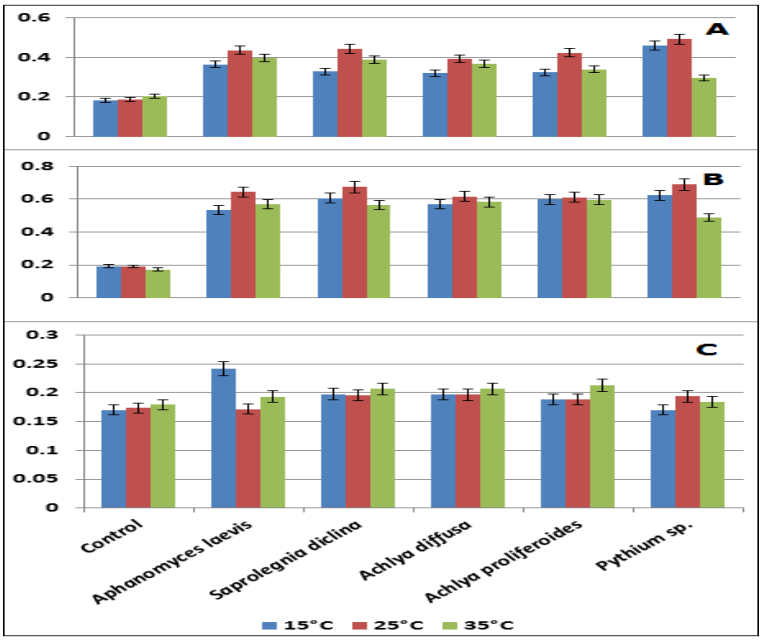

Fig. 8: Protease activity (specific activity µmol/mg-min) at pH 8.5 on different incubation period (A: 48 h; B: 72 h; C: 96 h)

The period of incubation had variable effects on protease production. At 48 h incubation period, 7.5 pH was more favourable than 6.5 and 8.5 pH ranges and similar was found after 72 h of incubation, but, 72 h incubation period was the best. However, at 96 h period of incubation the situation has been entirely different when the protease production was almost parallel to the control condition at all studied pH ranges (6.5, 7.5 and 8.5).

The study of pathogenesis on fish by the test watermoulds (*Saprolegnia diclina*, *Aphanomyces laevis*, *Achlya diffusa*, *Achlya proliferoides* and *Pythium oligandrum*) has shown an interesting observation that 90% fish infection has been recorded following 48-72h of incubation, duringcontrolled re-infection studies in the laboratory (using 10 fish specimens of *Labeo rohita* in triplicate), at an average temperature of 25^{o}C and at pH range of 7.5 which coincides with the maximum protease activity (0.70 µmol./mg-min) on these parameters (Fig. 9). This, therefore, distinctly indicates towards a direct correlation between protease activity of the watermoulds and their virulence during their pathogenesis on fishes. Furthermore, protease enzyme which hasinvariably been produced by pathogenic watermoulds has a critical

effect during pathogenesis on fishes (Fig. 10).

Saprolegnian infections (infections caused by watermoulds) of fish are frequently associated with the wounds and lesions and also that handling fish may predispose them to infection. The obvious inference, drawn from these observations is that these fungi act as "wound parasites". The integument (skin) of fish in general and the mucus, in particular, both present a physical and a biochemical barrier to the initiation of infection and that if this barrier can be breached, an infection can proceed unrestrained. This has already been discussed[6-8]. It does not appear likely that watermoulds produce any toxins[9-11]. The extent of damage caused by these fungi may directly be related to the tissue necrosis done by the hyphae in its surrounding area. Assuming that the fungus is the only pathogen, the time of death will be a function of the growth rate of the fungus, the initial site of infection, the type and quantity of tissue destroyed, and the ability of the individual fish to withstand the stress of the disease. However, production of certain pectolytic or proteolytic enzymes by these fungi cannot be ruled out. Scientists[11, 12] have suggested a correlation between the production of a chymotrypsin-like proteolytic enzyme and the capacity for an isolate of *Saprolegnia ferax* and four isolates in the *S. diclina-S. Parasitica* complex to switch over from a saprotrophic to a necrotrophic mode of nutrition. The present investigations support the contention that pectolytic and proteolytic enzymes are invariably produced by the pathogenic watermoulds and these have a critical role in deciding their virulence during pathogenesis on fish.

Maximum production of protease has been observed[13] at 9.0 pH in some bacteria, yeast and certain filamentous fungi (like *Aspergillus sp.*, *Penicillium sp*. and *Fusarium sp.*) isolated from the fruits of *Coffea arabica*. Mohanasrinivasan *et al.*[14] have also reported maximum protease activity of 307.5 mol. /ml-min at pH 9.0 after 96 hours of incubation in *Aspergillus niger*. The higher fungi and bacteria are sturdier and basophilic in nature as compared to the watermoulds. The present investigations on pathogenic watermoulds (lower fungi) have indicated maximum production of protease at 7.5 pH following 72 h of incubation and are reduced after 96 h incubation period.

CONCLUSION

The qualitative and quantitative production of protease enzyme by pathogenic watermoulds, viz., *Saprolegnia diclina*, *Aphanomyces laevis, Achlya diffusa, Achlya proliferoides* and *Pythium oligandrum*

has been assessed and a correlation between severity of infection and the protease production has also been determined. The observed data showed that there was maximum protease production by each test fungi at pH 7.5

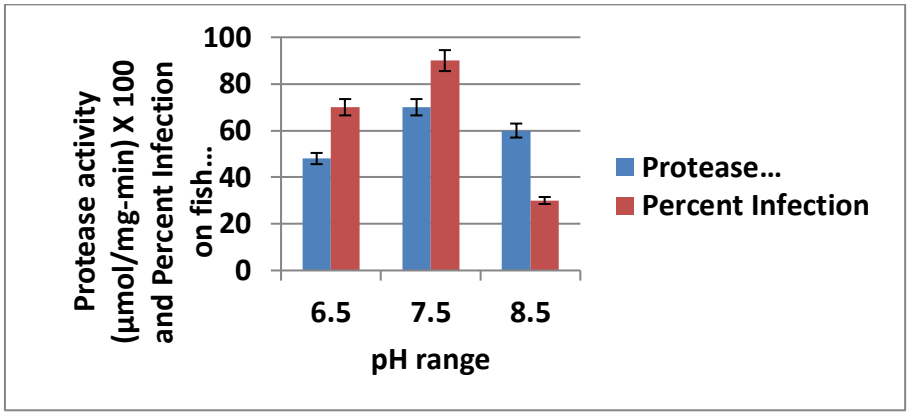

Fig. 9: Protease production by watermoulds and per cent infection on fish by watermoulds at 72 h of incubation period.

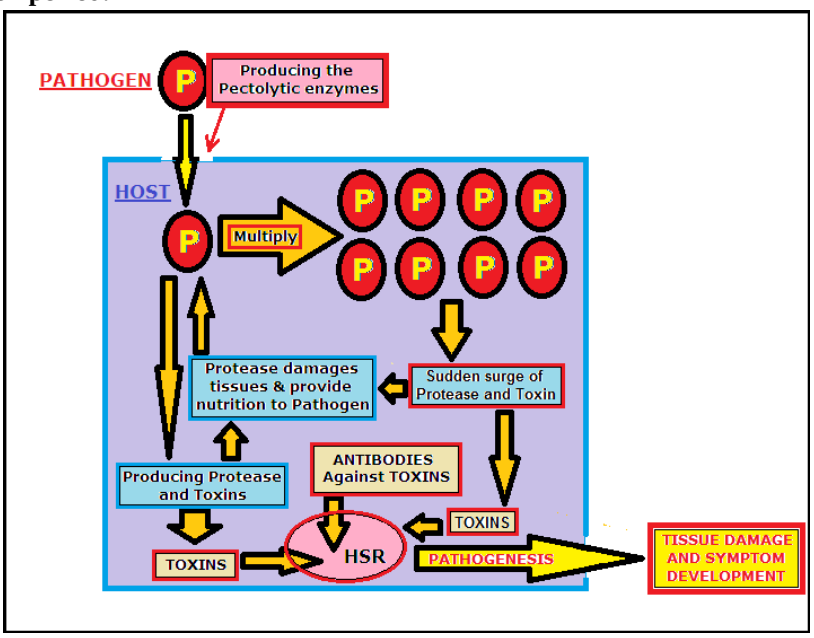

Fig. 10: Watermould pathogenesis on fish and the role of enzymes. P: Pathogen; HSR: Hyper Sensitive Reaction (after Srivastava *et.al.*,[16])

and at 25°C, while the slightly acidic pH 6.5 showed the minimum extracellular protease production. At 72 h incubation period maximum extracellular protease production has been observed by all the test fungi at 25°C and 7.5 pH range. The

present results distinctly indicate that extracellular protease enzyme is invariably produced by the pathogenic watermoulds and there has been a direct correlation between protease production bythe watermoulds and their virulence during their pathogenesis on fishes.

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