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Production of extracellular proteases by *Aeromonas, Serratia* and *Shewanella* species and characterization of extracellular proteases using zymogram analysis.

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Abstract: *Seafood spoilage microorganisms are known to produce extracellular proteases. The current research was aimed at using SDS-PAGE substrate impregnated gel to characterize the proteases secreted by the spoilage bacteria. Extracellular proteases (ECP) of seven spoilage microorganisms namely; Shewanella baltica OS185, Aeromonas sp. HB-6,, A. hydrophila HX 201006-3, Sh. baltica, Sh. baltica OS678, Serratia spp. I-113-31 and A. salmonicida subsp. achromogenes isolated from various seafoods were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Coomassie brillant blue-stain. Extracellular proteases of the seven spoilage microorganisms were characterized by substrate (gelatin and sheep blood) co-polymerized SDS-PAGE. The proteolytic activity results of the (ECPs) the spoilage microoganisms revealed the presence of many bands ranging in molecular weight from 20 to 100 kDa which represents serine, cysteine, aspartic and metallo proteases. The gelatinase profiles for Aeromonas spp., Shewanella spp. and Serratia spp. were characterized by the presence of high molecular weight metallo proteases ranging from 69, 70, 100, 110,140 and 220 kDa; 70, 100 and 120 kDa and 100 kDa, respectively. Whereas the sheep-blood profiles for Aeromonas spp., Shewanella spp. and Serratia spp. were characterized by the presence of serine, cystein, aspartic and high molecular weight metallo proteases ranging from 37, 50 and 100 kDa; 35, 37, 50 and 100 kDa and 44 kDa respectively.*

Keywords **-** *Aeromonas* spp., extracellular proteases, gelatin and sheep-blood zymogram, *Serratia* spp., *Shewanella* spp**.**

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I. Introduction

Generally, aeromonas disease in fish has been related with *Aeromonas salmonicida* and *A. hydrophila*, which are linked with furunculosis/ulcer disease and motile aeromonas septicaemia/generalized haemorrhagic septicemia/fin rot, respectively [1]. The virulence of *Aeromonas* is related to the production of a variety of extracellular toxins such as proteases, lipases, elastase, lecithinase, chitinases, and haemolysins [2]. Some proteolytic enzymes secreted by *Aeromonas* are known to be significantly involved in invasion and establishment of infections by overcoming initial host defenses and by providing nutrients for cell proliferation [3]. Two main proteolytic activities of motile *Aeromonas* has been recognized, a 65-68 kDa temperature labile serine protease [3], and a 38 kDa thermostable metalloprotease [4].

Serratia marcescens is extensively spread in natural environments and has appeared as an important nosocomial pathogen, mainly in immunocompromised patients [5]. Even though *S. marcescens* pathogenicity is not well understood, its extracellular secreted enzymes, including several types of proteases, are candidates for virulence factors [5]. Some workers have reported the distribution and multiplicity of proteases among different strains of *Serratia* [6]; [7] and characterization of extracellular proteases from different strains of *S. marcescens* [8] showing that most strains produce a very similar major metallo-protease.

Shewanella strain Ac10 has been cloned in *Escherichia coli*, the recombinant serine alkaline protease of *Shewanella* strain Ac10 was found to have a molecular weight of about 44 kDa and to be highly active in the alkaline region (optimum pH, around 9.0) when azocasein and synthetic peptides were used as substrates [9].

The objectives of the study were to describe the extracellular proteases (ECPs) produced by *Aeromonas, Serratia* and *Shewanella* species and to characterize the extracellular proteases complex produced by these spoilage organisms using SDS-PAGE gels co-polymerized with gelatin and sheep-blood.

II. Materials and Methods

2.1. Bacterial strains and culture

The bacterial strains investigated in this study were *Sh. baltica* OS185, *Aeromonas* sp. HB-6, *A. hydrophila* HX 201006-3, *Sh. baltica*, *Sh. baltica* OS678, *Serratia* spp. I-113-31 and *A. salmonicida* subsp. *achromogenes*. Isolates were obtained from fresh Atlantic salmon, smoked and fresh haddock collected from local shops Central in Scotland, were previously identified phenotypic traits and 16S rDNA sequencing [10].

2.2. Preparation of bacterial extracellular products (ECPs)

Preparing the spoilage bacterial ECPs were obtained by the cellophane plate technique [11]. Five ml of bacterial cultures were grown for 24 h at 30 ˚C on tryptone soya broth (TSB; Oxoid) supplemented with 1% (w/v) sodium chloride [=TNB], 1 ml of the bacterial culture was spread onto sterile Cuprophan sheets (Medicell) which had been layered onto on tryptone soya agar (TSA; Oxoid) plates supplemented with 1% (w/v) sodium chloride [= TNA] in a large petri dish (14 cm diameter). The bacteria were incubated for 48 h at 30 ˚C and the bacterial culture harvested by washing the cellophane with sterile PBS. The bacterial suspension was centrifuged at 3500 × g for 1 h, and the supernatant was then concentrated using 10,000 MW cut-off spin concentrators (Vivascience) by centrifuging at 2000 × g for 1 h. The supernatant was then filtered using 0.45-µm filter and aliquot and stored at -20 °C.

2.3. Sodium dodecylsulphate-polyacrylamide electrophoresis (SDS-PAGE)

The ECPs in cell free culture supernatants were separated by non-reducing SDS-PAGE on a Multiple Gel Caster (Hoefer) with 12.5% (w/v) acrylamide resolving gel (Next Gel™) (Amresco®) with modification of [12]. Combined volumes of the protein samples and SDS-PAGE sample buffer containing (0.5M Tris-HCl pH 6.8, 10% (v/v) glycerol, 10% (w/v) SDS, 0.003% (w/v) bromophenol blue and 0.3% (w/v) dithiothreitol (DTT)) in tube and placed in boiling water bath and heated for 2 min before application to the gel. The protein samples were cooled on ice until ready to use. The gel was polymerized chemically by the addition of 7.5 µl of tetra-ethylene-diamine (TEMED) and 75 µl of ammonium persulphate (APS). Five µl volumes of molecular weight marker and 15 µl of protein samples were loaded on the gel. Electrophoresis was carried out using electrophoresis power supply (EPS 1001) (G.E. Health care). Electrophoresis of samples was at 175V, 313mA, 100W, 1.5 h or until the sample was seen at the bottom of gel. Part of the gels were stained overnight with Coomassie Brilliant blue and silver stained [13] for the visualization of proteins Molecular weights were estimated by comparison with protein standards (Fisher # 26634, Spectra ™ Multicolour Broad range Protein Ladder).

2.4. Substrate-impregnated PAGE

The non-denaturing gel electrophoresis method of [14] modified by [15] was used, with minor modifications to detect and estimate the size of proteases present in the ECPs of the spoilage bacteria. Gelatin (Sigma-Aldrich) and sheep blood (Oxoid) were individually added each to 10% acrylamide gel at a final concentration of 0.2% protein (w/v). Volumes of the protein samples and native sample buffer containing (0.5 M Tris-HCl, pH 6.8, 10% (v/v) glycerol, 0.003% (w/v) bromophenol blue) were combined to give 600 µg ml^{-1} concentration of the ECP. The samples were not boiled before application to the gels since substrate gel electrophoresis depends upon the maintenance of proteolytic activities. Five µl volumes of the molecular weight marker and 15 µl of protein samples were loaded onto the substrate gel. Electrophoresis was carried out using Mighty Small SE245 system (Hoefer) at 4°C for 4 h, with a constant current of 70 volts, 313 mA and 100 W. After electrophoresis, the gels were then incubated overnight in 0.1 M glycine-NaOH, pH 8.3. Gels were then fixed and stained with Coomassie Brilliant Blue overnight, then destained until clear gel degradation halo bands were apparent where proteolytic hydrolysis of gel embedded substrate had occurred. Proteases were separated according to their different electrophoretic mobilities and detected based on their ability to digest the protein substrate incorporated into the acrylamide gels.

III. Results

3.1. SDS-PAGE identification of extracellular proteins of potential pathogenic and seafood spoilage microorganisms.

The results of SDS-PAGE identification of extracellular proteins of potential pathogenic and seafood spoilage microorganisms using Coomassie brilliant blue stained gel are shown in Figure 1. The profiles of the extracellular fractions of *Aeromonas* sp. determined by on SDS-PAGE Coomassie brillant blue-stained gel (Fig. 1.) clearly showed the presence of protein bands. With regard to *Aeromonas* sp. HB-6 bands (lane 2) were detected at 50, 70 and 80 kDa for Cooassie brillant blue-stained gel. The extracellular fraction of *A. hydrophila* HX 201006-3 (lane 3) in this study revealed the presence of protein bands at 20 and 35 kDa on Coomassie brillant blue-stained gel. The profile of the extracellular fraction of *A. salmonicida* subsp. *achromogenes* (Lane

7) clearly showed the presence of protein bands with molecular weights 20, 37 and 70 kDa. Prominent bands of the extracellular fraction of *Serratia* spp. I-113-31 (lane 6) were shown on Coommasie blue stain at 50 and 70 kDa. The profile of the extracellular fractions of *Sh. baltica* OS185, *Sh. baltica* and *Sh. baltica* OS678 (Fig. 1.) clearly revealed presence of protein bands with molecular weights 25, 35, 40 and 50 kDa (lane 1); 15, 37, 40, 50, 70 and 85 kDa (lane 4) and 25, 55 and 85 kDa (lane 5) for Coomassie brillant blue-stained gel, respectively. The proteolytic activity results of the (ECPs) the spoilage microoganisms revealed the presence of serine, cysteine, aspartic and metallo proteases.

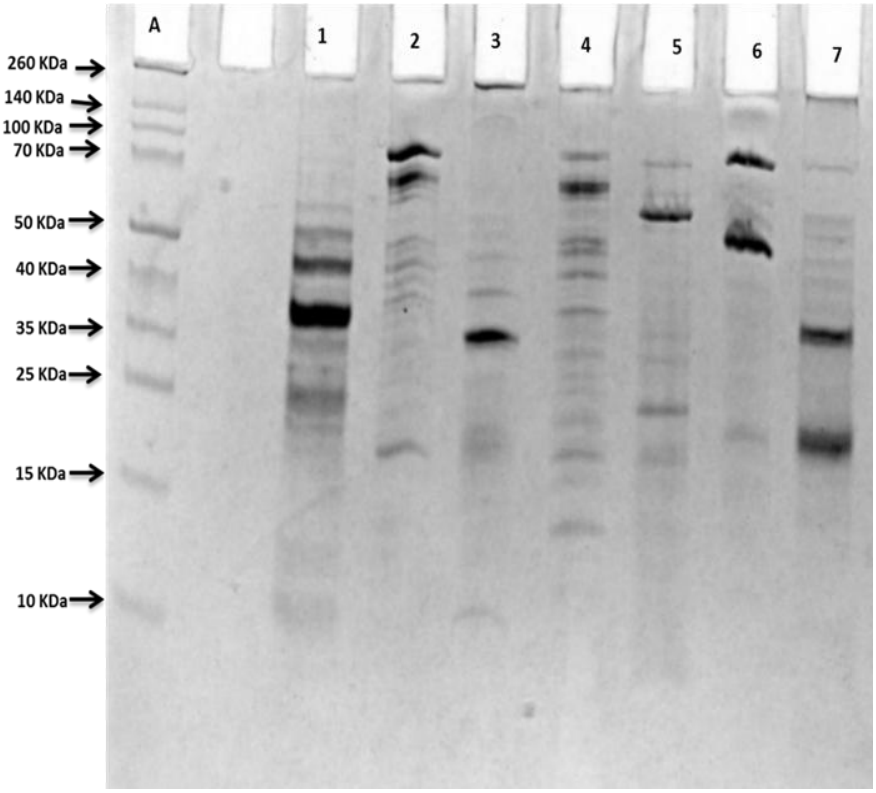

Figure 1. Coomassie brilliant blue-stained gel of extracellular proteins by potential pathogenic and spoilage microorganisms from seafoods as separated by SDS-PAGE. Lane A, Molecular weight standard markers; Lane 1, *Sh. baltica* OS185; Lane 2, *Aeromonas* sp. HB-6; Lane 3, *A. hydrophila* HX 201006-3; Lane 4, *Sh. baltica*; Lane 5, *Sh. baltica* OS678; Lane 6, *Serratia* spp. I-113-31; Lane 7, *A. salmonicida* subsp. *achromogenes*.

3. 2. Gelatinase and Haemolysin proteases of extracellular proteins of potential pathogenic and spoilage seafood microorganisms by substrate SDS-PAGE is shown in Figures 2 and 3.

Substrate-impregnated SDS-PAGE was used to investigate the substrate specificity of individual proteases. The ECPs of *Aeromonas* sp. HB-6, *A. hydrophila* HX 201006-3 and *A. salmonicida* subsp. *achromogenes* revealed bands with proteolytic activity by the analysis of gelatin co-polymerized SDS-PAGE patterns (Fig. 2.). Gelatinase patterns in the *Aeromonas* species were characterized by the presence of bands of high MW at 70 kDa, 69 and 100 kDa, and 110, 140 and 220 kDa, respectively.

The zymogram assay of the haemolytic patterns of *Aeromonas* sp. HB-6, *A. hydrophila* HX 201006-3 and *A. salmonicida* subsp. *achromogenes* in sheep blood showed a band at MW of 37 kDa, 100 kDa and 50 kDa, respectively (Fig. 3.). The protease (gelatin) pattern of *Serratia* spp. I-113-31 was demonstrated at approximately 100 kDa (Fig. 2.), while the haemolysin pattern was revealed at 44 kDa (Fig. 3.). Zymogram analysis of the *Sh. baltica* strains using gelatin revealed different molecular masses of approximately 100 kDa for *Sh. baltica* OS185, 70 and 120 kDa for *Sh. baltica* and 120 kDa for *Sh. baltica* OS678 (Fig. 2.). The haemolytic pattern showed different molecular weights of approximately 50 kDa for *Sh. baltica* OS185, 37 kDa for *Sh. baltica* and 35, 50 and 100 kDa for *Sh. baltica* OS678 (Fig. 3.).

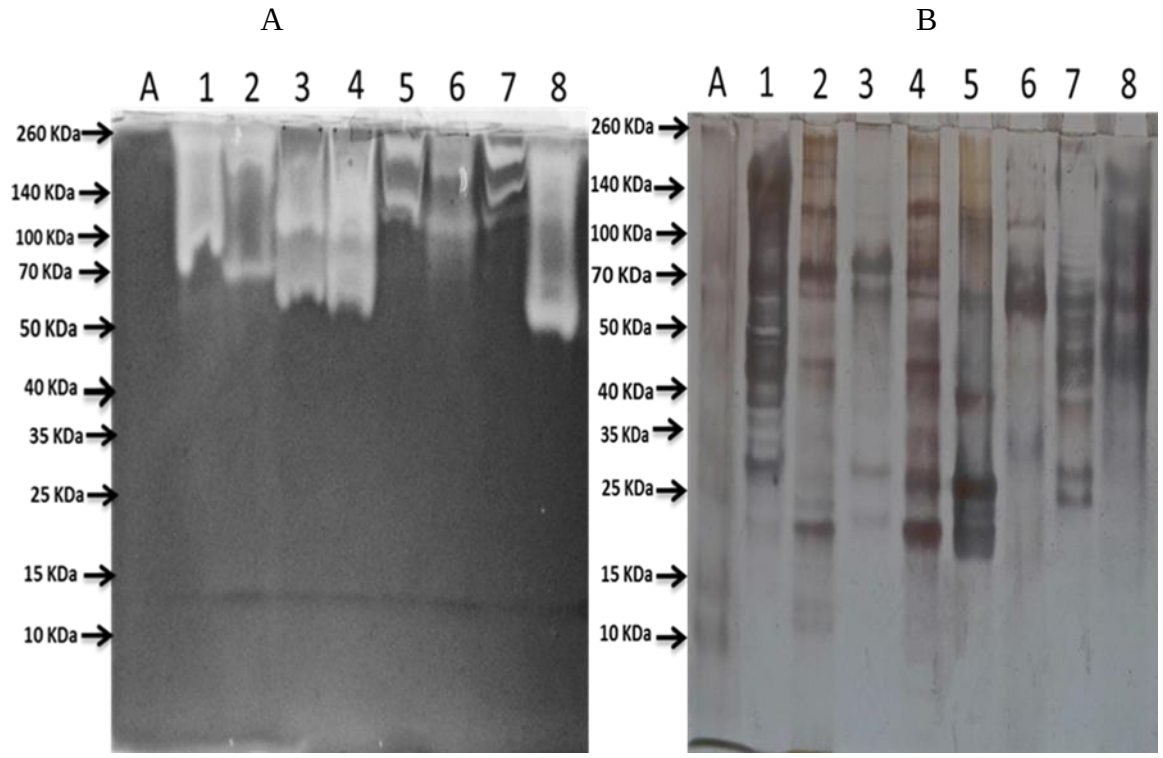

Figure 2. Zymogram assay. Protease pattern in gelatin resolved by native gel electrophoresis analysis was carried in acrylamide gel (10%) to express protease from various extracellular protein by potential pathogenic and spoilage microorganisms from seafood. Lane A, Molecular weight standard markers; Lane 1, *Sh. baltica* OS185; Lane 2, *Aeromonas* sp. HB-6; Lane 3, *A. hydrophila* HX 201006-3; Lane 4, *Sh. baltica*; Lane 5, *Sh. baltica* OS678; Lane 6, *Serratia* sp. I-113-31; Lane 7, *A. salmonicida* subsp. *achromogenes*; Lane 8, Trypsin (Control). (Panel A) Gelatin zymogram, (Panel B) Silver stained gel.

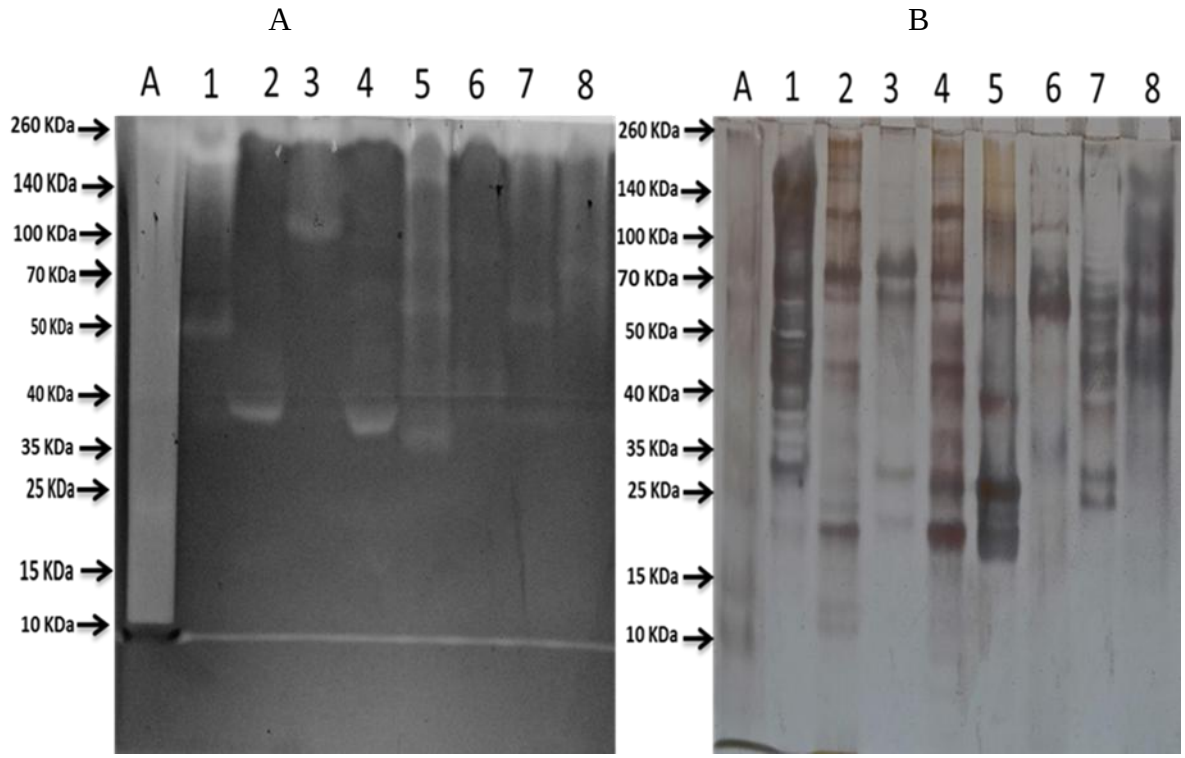

Figure 3. Zymogram assay. Haemolysin pattern in sheep blood resolved by native gel electrophoresis analysis was carried in acrylamide gel (10%) to express protease from various extracellular protein by potential pathogenic and spoilage microorganisms from seafood. Lane A, Molecular weight standard markers; Lane 1, *Sh. baltica* OS185; Lane 2, *Aeromonas* sp. HB-6; Lane 3, *A. hydrophila* HX 201006-3; Lane 4, *Sh. baltica*; Lane 5, *Sh. baltica* OS678; Lane 6, *Serratia* spp. I-113-31; Lane 7, *A. salmonicida* subsp. *achromogenes*; Lane 8, Trypsin (Control). (Panel A) Sheep blood zymogram; (Panel B) Silver stained gel.

IV. Discussion

[16], [9] and [17] determined the molecular weights of the ECPs of the *S. marcescens*, *Shewanella* strain Ac10 and *A. hydrophila* strain An4, respectively using SDS-PAGE gels, whereas [18] used substrate-impregnated SDS-PAGE for investigating the substrate specificity of individual proteases of *Aeromonas* species. From the present work, it was revealed that the proteases of spoilage microorganisms are grouped into different protease groups.

The molecular mass of bacterial hemolysins is within the range of 49 to 60 kDa [19]; [20]. This study has shown the presence of a prominent protein band at 50 kDa in an extracellular protein fraction of *Aeromonas* sp. HB-6 which may represent the presence of the bacterial toxin haemolysin [21]; [17] *Aeromonas* spp. HB-6 protease on SDS-PAGE revealed prominent bands at 50, 70, and 80 kDa which represents aspartic protease, metallo protease and cysteine protease (μ calpain), respectively [22]; [23]; [24]. Zymograms obtained on gelatin and sheep blood co-polymerized SDS-PAGE allowed identification of bands at 70 and 37 kDa, respectively, for *Aeromonas* spp. HB-6. Such serine proteases (subtilisin-like) having molecular weights ca. 60-70 kDa have been defined in *A. hydrophila* and *A. sobria* [25]; [26]; [3]; [27]. These proteases designated as temperature labile have been branded as virulence factors with high caseinolytic activity, and implicated in elastase maturation (i.e the formation of two disulphide bonds present in the mature enzyme) [4]; [3]. The protease activity coupled with haemolytic activity shown by *Aeromonas* sp. HB-6 further strengthens its pathogenic capability as it has already been ascertained that the presence of both proteins increases the intensity of the virulence of this fish pathogen [28].

A. hydrophila HX 201006-3 revealed prominent bands at 20 and 35 kDa on SDS-PAGE which represents serine (trypsin) protease respectively [23]; [29]. Two bands with proteolytic activity were detected at 69 and 100 kDa by analysis of gelatin co-polymerized and 100 kDa sheep blood co-polymerized SDS-PAGE for *A. hydrophila* HX 201006-3. [18] detected high molecular weight metalloprotease bands (AMW 83 and 94 kDa) with gelatin co-polymerized SDS-PAGE patterns particularly prevalent among *A. hydrophila* strains.

A. salmonicida subsp. *achromogenes* showed prominent bands at 20 and 37, and faint band at 70 kDa. [30] reported the presence of 22 kDa proteases produced by three strains of *A. salmonicida* which are of lower molecular weight (probably around 20,000 Da) which are active against gelatin but not casein. The faint band revealed at 70 kDa for *A. salmonicida* subsp. *achromogenes* corresponding to the virulence protein, P1 protease of *A. salmonicida* typical strains (70 kDa serine protease) which has been demonstrated alone to produce muscle liquefaction and haemorrhages when injected i.m. [31]. [32], [33]) demonstrated the presence of 70 kDa protease band that is a caseinolytic protease produced by three strains of *A. salmonicida.* The function of 70 kDa serine-protease which is the most abundant protein in the ECP is the digestion of host proteins (used as a nutrient source), thus facilitating the colonization of the host and enhancing the pathogenicity of the bacterium. [34] reported that band at 37 kDa may probably correspond to the metalloprotease as judged by its inhibition of metal chelating agents. Zymograms obtained on gelatin and sheep blood co-polymerized SDS-PAGE (Figs. 2 and 3) allowed identifying bands at 110, 140, 220 kDa and 37 and 50 kDa respectively for *A. salmonicida* subsp. *achromogenes*. [18] demonstrated the presence of gelatinolytic patterns with distinctive bands of high molecular weight metalloproteases of (73, 83 and 94 kDa) in *Aeromonas* species. The results reported in the present work identified high molecular weight gelatinases in *A. salmonicida* subsp. *achromogenes*. [34] identified high molecular weight gelatinases in several *A. salmonicida* strains zymograms. The molecular weights obtained from the results here are higher than that of [34], but several other proteolytic factors [35] have not so far been associated with *A. salmonicida* pathogenesis. [36] reported that some protein-protein complexes are not dissociated in zymography. The study of protease complexes of various organisms, including *A. salmonicida* has been demonstrated using zymogram as a sensitive and dependable method [34]; [37].

Gudmundsdóttir *et al*. (2003) [37] reported that the ECPs of two *A. salmonicida* subsp. *masoucida* type strains and one atypical isolate were found to possess pronounced haemolytic activity, but not detectable phospholipase activity, were just under the limit (A_{450} = 0.4) to be defined as weakly positive (A_{450} = 0.34-0.37) in the GCAT-ELISA. Furthermore, analysis of the same three ECPs revealed stronger 60 kDa bands in zymograms than those of other GCAT negative strains. In contrast with the result of our study the zymogram assay of the haemolytic pattern of *A. salmonicida* subsp. *achromogenes* in sheep blood showed a band at MW of 50 kDa.

Two prominent bands with proteolytic activity were detected at 50 and 70 kDa on SDS-PAGE analysis of *Serratia* spp. I-113-31, representing aspartic protease and metalloprotease respectively [22]; [23]. [16] demonstrated the molecular weight of purified extracellular, nonelastolytic, neutral metalloprotease of *S. marcescens.* Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of the denatured protease preparation gave a molecular weight of approximately 52.5kDa. The work of [38] demonstrated the production of purified four distinct proteases from culture filtrate of *S. marcescens* kums 3958, a fresh isolate from a patient with a severe corneal ulcer. These proteases marked 56K, 60K and 73K. Only the 56K and 60K proteases had molecular weights similar to the previously reported serratial proteases; that is the molecular weights of proteases from *S. marcescens* ATCC 25419 [39], strain BG [16] and *S. piscatorum* [40] were 51.9, 52.5 and 60 kDa, respectively. This research work revealed bands at approximately 50 and 70 kDa showing the proteolytic activity of *Serratia* spp. I-113-31. The work of [16] revealed the zymogram analyses of purified extracellular, nonelastolytic, neutral metalloprotease of *S. marcescens,* patterns revealed the protease activity as microheterogenous. They estimated the apparent molecular weight of the native enzyme, by Sephadex G-100 gel filtration at approximately 44 kDa. In contrast, to the present work this demonstrated protease (gelatin) pattern of *Serratia* spp. I-113-31 at approximately 100 kDa. The reason for the variation in molecular weight might be due to unpurified extracellular product and also use of a different protein substrate. Furthermore, the present research work revealed the zymogram pattern of haemolytic activity of *Serratia* spp. I-113-31 at 44 kDa. In contrast [41] reported that the molecular weight of ShlB which habours the *S. marcescens* haemolysin determinant was 62 kDa.

Four bands with proteolytic activity were detected at 25 and 35, 40 and 50 kDa on SDS-PAGE analysis *Sh. baltica* OS185. They represent serine protease and aspartic protease respectively [22]; [29]. Bands with proteolytic activity were detected at 100 and 50 kDa, respectively by analysis of gelatin and sheep blood co-polymerized SDS-PAGE for *Sh. baltica* OS185. SDS-PAGE analysis of *Sh. baltica* analysis of revealed proteolytic activity at 15 ,37, 40, 50, 70 and 85 kDa which represents aspartic proteases, metallo proteases and cysteine proteases, respectively [22]; [23]; [24]. Zymograms obtained on gelatin and sheep blood co-polymerized SDS-PAGE allowed identifying bands at 70 and 120 kDa and 37 kDa respectively. Three bands with proteolytic activity were observed at 25, 55 and 85 kDa on SDS-PAGE anlysis *Sh. baltica* OS678. Bands with proteolytic activity were detected at 120 kDa and 35, 50 and 100 kDa respectively for by analysis of gelatin and sheep blood co-polymerized SDS-PAGE for *Sh. baltica* OS678. The ECPs of *Shewanella* strains are all grouped into the different protease groups. The extracelluar products of *Sh. baltica* and *Sh. baltica* OS678 revealed protein bands of MWs approximately 85 kDa in the present study. [9] reported a molecular weight of 44 kDa serine alkaline protease (SapSh) of psychrotrophic bacterium *Shewanella* strain Ac10 cloned with *E. coli* when azocasein and synthetic peptides were used as substrates. Differences in molecular weights may be due to use of different substrates.

V. Conclusion

The virulence of bacterial pathogens has been associated with the production of extracellular proteases. This current study revealed that proteases of spoilage microorganisms are grouped into different protease groups.

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