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Isolation and characterization of protease producing bacteria from upper respiratory tract of wild chicken

Sangeeta Raut[1], Sudip Kumar Sen[1]*, Naveed Alam Kabir[1], Soumya Satpathy[1], Smita Raut[2]

[1]Department of Biotechnology, Gandhi Institute of Engineering and Technology, Gunupr,Rayagada,Odisha-765022,India; [2]M.S Swaminathan Research Foundation, Jeypore, Koraput, Odisha, India; Sudip Kumar Sen - Email: sudip.bio@gmail.com; Phone- +91 9437505507; *Corresponding author

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Abstract:
Bacterial samples isolated from the upper respiratory tract of a healthy broiler chicken and a wild chicken suffering from influenza which were collected locally revealed proteolytic activity as detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram analysis. Among five protease producing strains screened, one was selected as promising protease producer. The activity of the protease produced by this organism is stable up to 620C. Optimum yield was achieved after 19 hours of culture, at pH 9.0 and 450C. The desired protein was precipitated from the crude extract by using ammonium sulfate (60%) followed by dialysis and purified by Ion-exchange chromatography. Further investigations are needed to know about the structure elucidation of the purified protein for industrial exploitation.

Keywords: Protease, SDS-PAGE, Zymogram, Gunupur

Background:
The role of alkaline proteases in facilitating the host cell entry by influenza A viruses through receptor mediated endocytosis is now well documented **[1].** Evidences are mounting that extracellular proteases are responsible for the proteolytic cleavage activation of avirulent avian and mammalian influenza viruses and contribute to pathogenicity and tissue tropism **[2]**. As a matter of fact, the cleavage of HA of virus allows into HA1 and HA2 the exposure of amino-terminal fusion peptide of HA2 within the endosome, which in turn facilitate the fusion of viral envelope with the endosomal membrane.

The sources of specific proteases that confer cleavage activation of influenza A viruses, however, is yet to be fully understood. While intracellular proteases recovered from nasal wash samples of children suffering from upper respiratory disease were able to cleave HA of influenza virus, several endogenous host inflammatory and hemostasis proteases such as kallikreine, urokinase, thrombin and plasmin, etc., were found to be capable of cleavage activation of only a few, but not all, influenza viruses. Similarly, several other host proteases such as granulocyte proteases and cathepsin D were found to cleave the HA but were incapable of conferring infectivity **[3]**. Contrary to this, staphylokinase, streptokinase and a protease isolated from *Serratia marcescence* was found to facilitate cleavage activation by generation of plasmin from plasminogen **[4]**,where as a protease namely elastase secreted by *Pseudomonas aeruginosa* isolated from seven out of 11 proteolytic bacteria isolated from nasal samples of swine could cleave influenza HA viruses directly.

The proteases obtained from bacteria present in the respiratory tract revealed a similar picture. The proteases recovered from several strains of *Streptomyces griseus*, *Staphylococcus aureus* and *Aerococcus viridians* could cleave some but not all influenza A viruses, though HA cleavage by *S.aureus* and *A.viridias* could confer infectivity as well as replication both *in vitro* and mice *in vivo* **[5, 6]** . Highly proteolytic bacteria namely *Flavobacterium* sp. and *Vibrio alginolyticus* which are said to be associated with soil and water sources have also been isolated from respiratory tract of poultry birds. Certain bacterial species namely *Staphylococcus*

hyicus, E.coli and *Streptococcus sp.* have also been reported to cause acute fibrinopurulent blepharitis and conjunctivitis, in chickens and turkeys **[7]** *S. huicus* in particular has been found to play a role in inducing during other diseases like pox and joint asteomyelitis **[8].**

Thus variations in the number of proteolytic isolates and the degree of proteolytic activity as seen in pigs as other mammals, has also been observed in the upper respiratory tract of poultry **[9].** Based on these observations, it has been suggested that proteolytic environment of nasal mucosa and mucous membrane cells of other digestive tract may differ between animals and that such variability may account for some of the differences in disease severity. It appears, therefore, likely that examination of proteolytic bacteria from nasal flora of poultry and chicks especially from nasal specimens as well as specimens suffering from influenza viral infection may prove regarding in understanding the contribution of endogenous and microbial proteases in natural influenza virus infection. The present paper describes the protease activity of one out of five bacteria isolated from the upper respiratory tract of one locally collected poultry with no sign of influenza and another wild chicken suffering from influenza virus. The protease activity of the flora was assessed and the single species showing protease activity was analysed

Methodology:

Site of isolation and cultivation conditions

Bacteria were collected from the upper respiratory tract of one healthy broiler chicken and another wild chicken suffering from influenza. The isolates were cultured in nutrient agar medium containing peptone 0.5 mg, Sodium chloride 0.5 mg, Beef extract 0.05 mg, Yeast extract 0.15.mg, Agar1.5 mg per 100mL of double distilled water. The colonies obtained were cultured in Gelatin agar medium with congo red as dye(selective media) composed of Gelatin 1 gm/100ml, Mannitol 1 gm/100ml, K_2HPO_4 0.05gm/100ml, NaCl 0.01 gm/100ml, $MgSO_4$ 0.02 gm/100ml, Yeast extract 0.01 gm/100ml, Agar 2 gm/100ml, Congo red dye 2 gm/100ml at pH 7.2. The colony obtained was found to be protease producing one and was sub-cultured in Gelatin yeast extract glucose broth composed of Gelatin 1 gm/100ml, glucose 1 gm/100mL, Yeast extract 0.2 gm/100mL, Di-potassium hydrogen phosphate 0.3 gm/100mL, Potassium di-hydrogen phosphate 0.1 gm/100mL, $MgSO_4.7H_2O$ trace amount at pH 7.2.

Determination of Optimum Growth Conditions

The optimum temperature for better growth was determined by incubating the culture at different temperature ranging from 35^0 C – 60^0 C in water bath shaker at 150 rpm for a period of 20hr. To determine the optimum pH for the growth, the culture was grown in Gelatin broth of different pH ranging from 5 to 10. The growth was defined in terms of Optical Density at 660nm (UV Vis spectrophotometer) **[10].**

Morphological characterization of proteolytic strain

The preliminary morphological characteristics were determined by using bright field microscopy (Zeiss Axiostar Plus microscope) following post simple staining with crystal violet dye .Gram staining, acid fast staining, endospore, capsule and flagellar staining were performed for cell surface analyses **[11]**.

Biochemical characterization

For biochemical characterization of proteolytic strain, tests like Methyl Red Test, Indole Production Test, Nitrate Reduction Test, Voges Proskauer Test and Citrate Utilization Test were performed as standard protocols.

16S rDNA Sequence Analysis

Sequence analysis of 16S rDNA (which occur as a conserved molecule in the bacterial domain) extracted using HELINI Biomolecules, Chennai, India. Kit formed the basis of molecular identification of the isolate **[12]**. The 16S rDNA sequences obtained were subjected to BLAST and were submitted to GenBank. The phylogenetic analysis was done by Neighbor Joining method.

Protease assay

The bacterial culture was centrifuged at 5000 rpm for 6 mins and 0.15 ml of the supernatant was added to a tube containing 0.3 ml of 1% (w/v) casein (dissolved in 20 mM Tris-HCl buffer, pH 7.4) and incubated at 37^oC for 30 mins. Subsequently, 0.45 ml of a 10% (w/v) tri-chloroacetic acid solution at a final concentration of 5% w/v was added to stop the proteolysis. The mixture was incubated at room temperature for 1 h. After incubation, the reaction mixture was centrifuged at 12000 ×*g* for 5 mins and the absorbance of the supernatant was measured at 280 nm. One unit of protease is defined as the amount of enzyme that hydrolyses casein to produce equivalent absorbance to 1 µmol of tyrosine/min with tyrosine as standard **[13].**

Preparation of Tyrosine standard curve

The concentration of protease secretion was estimated using tyrosine standard curve. The steps involved are same as described above for the protease assay except that instead of taking 3 mL of culture filtrate, 200, 400, 600, 800 and 1000 µg of tyrosine was taken in five separate test tubes along with 1 % of 3 mL caseine and 3 mL of phosphate buffer. Thereafter the absorbance was measured at 650 nm, taking Folin ciocalteu's reagent as control.

Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Zymogram Test

SDS-PAGE was carried out using a 12.5% (w/v) running gel by the method of Laemmli *et al*., 1979. For zymography, 0.5% (w/v) gelatin was co-polymerized with the running gel and samples were then loaded onto the gel without heating followed by electrophoresis with 100 V at room temperature. Following electrophoresis, the gel was incubated in 2.5% (v/v) Triton X-100 (renaturing solution) for 30 mins at room temperature with gentle agitation. The solution was then replaced with developing buffer (50 mM Tris, 0.2 mM NaCl, and 5 mM $CaCl_2$, pH 8.0) twice and the gel was incubated for 30 mins at 37^0C for 6 h. The gel was stained with Coomassie Brilliant Blue G-250 for 45 mins and then destained in double distilled water.

Results and Discussion:

Morphological and Biochemical Characterization of Isolates

The morphological characteristic of the proteolytic strain is illustrated in **Table 1 (see supplementary material).** Biochemically, the isolate was found to be positive for Gram's stain, Endospore stain, Catalase test, Starch hydrolysis, Voges

Prokauer test and Citrate utilization test but negative for Methyl red and Indole tests.

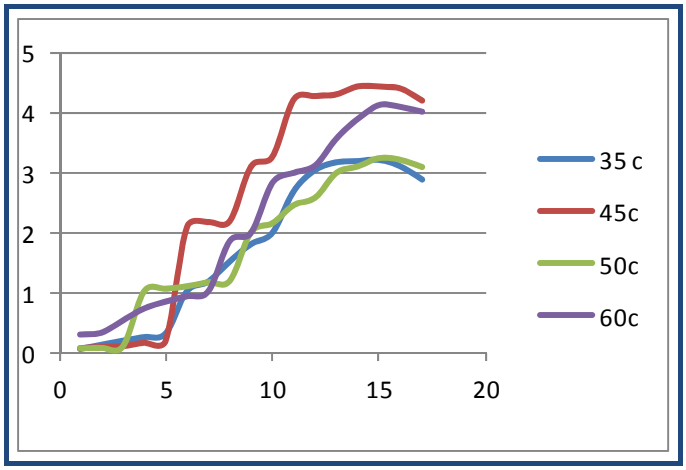

Figure 1: Depicting the growth profile of the isolates in enriched medium (LB broth). X-axis is time in hours and Y-axis is optical density.

Physiological Characterization

The strain showing maximum proteolytic activity designated as SRG was found to grow at temperature ranging from 20^{o}C to 60^{o}C. However, the optimum temperature for maximum growth was found to be 45 ^{0}C **(Figure 1)**.

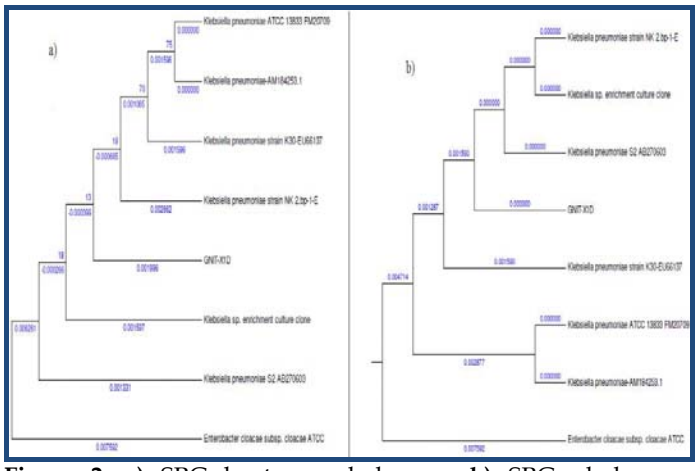

Figure 2: a) SRG bootsrap phylogeny; **b)** SRG phylogeny upgma

16s rDNA sequences analysis

The 16S rDNA based molecular phylogeny indicated that isolate (SRG) obtained was novel and hence submitted to GenBank which is available under the Accession No JN981163. Bootsrap and UPGMA phylogenies as predicted according to neighbor joining method are shown in Fig.2a and 2b. In both the types of phylogeny our strain (SRG) was found to have high sequence similarity with *Klebsiella pneumonae* strains NK 2bp- IE, S2AB 270603 and K- 30 EU 66137 **(Figure 2a & 2b).**

Determination of Protease Production

Polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram assays revealed a clear band of protease at around 60 kDa **(Figure 3a).** The concentration of protease was found to be around 750µg/mL as compared to Tyrosine Standard Curve **(Figure 3b).**

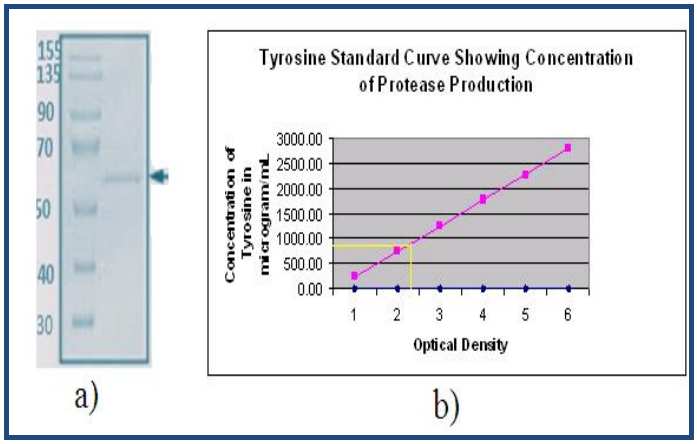

Figure 3: **a)** Protease from SRG; **b)** Tyrosine Standard Curve showing after Coomassei Brilliant Blue concentration of protease production.

Polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram analysis effect of pH on protease production

The effect of pH on protease activity of the isolated strain SRG was determined using fermentation media containing phosphate buffer (pH 5 – 6.0), Tris-HCl buffer (pH 7.0 - 8.0) and glycine-NaOH buffer (pH 9-10). The protease activity was measured and monitored at 6 hours intervals for a 72 h fermentation period through assay of protease activity. The maximum protease activity was found at pH 9 thereby indicating that the enzyme was active in alkaline condition **(Figure 4a).**Analysis of protease activity at varying temperature revealed that a maximum of 2µg/mL protease was produced by the SRG studied herein **(Figure 4b).**

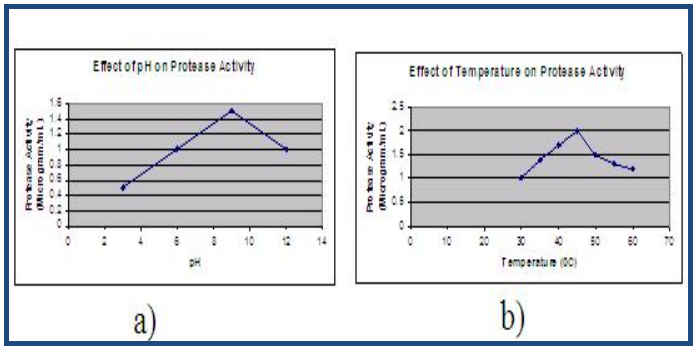

Figure 4: a) Effect of pH on Protease Activity**; b)** Effect of Temperature on Activity

Prior to this study a number of proteolytic bacteria have been isolated from the digestive and respiratory tracts of poultry chickens **[14].** A cursory survey of the data would make it evident that more than 82% of the birds studied contained at least one protease secreting bacterium and 50% had two or more protease secreting bacterial species in their lower digestive tract. Again, of the 20 protease secreting bacterial species screened by them 17 were gram positive cocci, two gram positive bacilli and only was gram negative bacillus.

Thus, the presence of one gram positive protease secreting coccus in one of our samples from upper respiratory tract of

country chicken is not surprising. In fact, occurence of proteolytic bacteria colonizing the respiratory tract of commercial/ country chickens appear to be a common phenomenon **[9].**

According to King *et al.,* 2009 the amount of protease secretion by proteolytic isolates is said to vary with growth of bacteria as well as with the substrates used for detection of proteolytic activity. Indeed, isolates studied by them did not secrete any detectable proteolytic enzyme in broth culture though all the isolates produced significantly large size clearing zone on casein agar assay as compared to known trypsin standard .We have not analysed the proteolytic activity of our isolate using casein agar assay, our results on concentration of protease produced by the strain studied however, using tyrosine standard clearly revealed that around 750 µg of protease was produced per ml. It appears, therefore, logical to suggest that the isolate (SRG) is an active protease secreting strain.

Our analysis of 16S r-DNA revealed that our strain (SRG) had close proximity to the *Klebsiella pneumoniae* sps. This was found in both the neighbor joining method. It appears therefore that our strain may turn out to be a novel species. This however needs to be confirmed through DNA sequencing. Microbial proteases are classified into various groups dependent on whether they are active under acidic, neutral on alkaline conditions and depending on the characteristics of the active site group , the enzyme is metallo- , aspartic- , cysteine , sulphdryl- or serine- type **[15, 16]**. Alkaline proteases are defined as those proteases which are active in a neutral to alkaline pH-range. The protease secreted by SRG strain studied here had highest activity at pH 9 thereby indicating that the protease belong to alkaline type. Further support to this supposition comes from our data on molecular weight of the enzyme (60kDa) and its stability up to 62^0C. However, as we have neither studied its substrate specificity nor its active sites based on biochemical assays or DNA sequencing we refrain from commenting whether it is seine type or any other type i.e. subtilases, carboxypeptidase, etc.. A further study in this direction is, therefore, warranted before suggesting any commercial application of the protease produced by SRG strain studied herein.

Conclusion:

Proteases play a critical role in almost every aspect of biology. Therefore a better understanding of their complex functions and regulation is vital to disease prevention and treatment, one of the first steps when investigating novel proteases are to establish its substrate specificity and a robust assay.

Here we have concluded that the isolated strain SRG has potential to produce alkaline protease. The highest protease activity was achieved at pH 9.0 and temperature at 45^0C. The molecular weight of the purified alkaline protease was found to be 60kDa. It is also very important to obtain enzymes with high stabilities and activities under alkaline pH and high temperature. Further investigations are needed on genetic analysis of this strain and structure elucidation of the purified protein for industrial exploitation. Additional manipulation of growth conditions may result in yields suitable for commercial applications.

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Supplementary material:

Table 1: The morphological characteristics of the proteolytic strain

Isolate	Cell morphology	Gram nature	Acid-fast staining	Capsule staining	Endospore staining	Flagella staining
SRG	Cocci	Gm+ve	-Ve	+Ve	+Ve	-Ve