JOURNAL OF CRITICAL REVIEWS

ISSN- 2394-5125 VOL 7, ISSUE 11, 2020

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoretic Analysis of a Potential Biocatalyst Screened from Swampy Milieu

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Abstract

SDS-PAGE (Sodium Dodecyl SulphatePolyacrylamide Gel Electrophoresis) is an important electroanalytical technique that has been conventionally used to separate proteins with different molecular masses. This electrophoretic separation process utilizes the combination of Sodium dodecyl sulphate (also known as Sodium lauryl sulphate) and Polyacrylamide gel to eliminate the influence of structure and charge and separate the proteins only on the basis of differences in their molecular weight.

Zymography is an electrophoretic method for the detection of proteolytic activity, based on the substrate repertoire of the enzyme. It is a singlestep staining method that enables the visualization and reliable quantitation of proteolytic activity for determining the approximate size of proteins in a sample on the basis of their hydrolysis of a protein substrate within the gel.

Silver staining is one of the most reliable and sensitive methods employed for protein analysis. Involving the deposition of metallic silver on gel surface at the location of protein bands.

The present paper describes the electrophoretic analysis of a sediment derived biocatalyst that can be potentially utilized in selected chemical transformations.

Key Words: Biocatalyst, Electrophoretic, Sediment, Silver staining, Zymography

INTRODUCTION

SDS - PAGE is the classical technique of qualitative analysis of a protein mixture used for monitoring protein purity and determining its molecular weight.It is a discontinuous electrophoretic system originally developed by Ulrich K. Laemmli[1]wherein the separation is based on the relative mobility of charged species towards the opposite electrode under the influence of an external or applied electric field through highly cross - linked surrounding matrix acting as a molecular sieve and retarding the movement of particles. The opposing interactions of electrical impetus and molecular sieving results in different migration velocities of molecules based on sizes, shapes and net charges. These migration rates are essentially a function of the charge/ mass ratio of the molecules being separated.

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Zymography is an experimental technique used for the detection and analysis of specific enzyme activity, in-situ, after electrophoresis. Since the method is based on SDS - PAGE, it involves the substrate copolymerized with polyacrylamide gel. Heussen and Dowdle devised an ingenious SDS - PAGE zymogram method to overcome the problem of protein denaturation. [2]In this method, the proteinaceous mixture is allowed to combine with SDS without boiling the solution. Thereafter, the electrophoretic separation of this SDS - protein mixture is achieved using a polyacrylamide gel containing low gelatin concentration. Proteinase - SDS complex migrates as a narrow band without binding with the constituent gelatin. The subsequent incubation in a non - ionic detergent namely Triton X-100 eradicates SDS from the complex thereby reconstituting the activity of proteinases. The strategic parts of gelatin are digested away by the reactivated proteinase and their positions can be ascertained by staining the gel. The digestion of gelatin appears as a clear band on the blue stained gel.[3]Casein and Gelatin Zymography have been routinely employed to investigate the purity and proteolytic activity of proteases. [4]

Silver staining is considered as an ultra-sensitive methodemployed for determining the total protein content at nanogram level sensitivity. It involves the deposition of metallic silver onto the surface of the gel at the location of protein bands. It combines excellent sensitivity whilst using simple economical equipment and chemicals and is also compatible with downstream processing such as mass spectrometric analysis post protein digestion.

This paper describes the electrophoretic characterization of a potential biocatalyst derived from swampy milieu that can be potentially utilized in selected chemical transformations.

EXPERIMENTAL STUDIES

Samples for the isolation of protease producing microbes were procured from marshy mangrove areas of Panjim, Goa. Twenty samples, labelled from B_1 to B_{20} were possessed for the isolation of bacteria and screened for their protease engendering potentialities.The morphological characteristics of bacterial colonies on the plate were recorded. These involved size, shape, color and consistency of the isolated colonies.In qualitative analysis during screening, 15 out of 20 cultures tested positive for protease production. Out of 15 positive cultures, 05 exhibited only gelatinase activity while 02 displayed only caseinase activity. Two media containing casein and gelatin as the protein substrate were studied for the two selected positive cultures B_2 and B_3.Data further proposed that isolate B_3 was most proficient in its proteolytic activity followed by B_2.

Since the isolate B_3 showed considerably higher activity for both the substrates, it was selected for further investigations.The bulk sample production from the most proficient B_3isolate was carried out using shake flask fermentation method followed by centrifugation and finally microfiltration. The extracted crude sample was concentrated 10 fold through Tangential Flow Filtration method and used for further studies.Optimization, Stability & Kinetic studies were performed. The sample was then subjected to the electrophoretic characterization.

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Characterization by Electrophoretic Method

Sodium Dodecyl Sulphate Polyacrylamide GelElectrophoresis (SDS-PAGE)[1]

Molecular weight of a protein sample was measured by subjecting the sample to electrophoresis in presence of SDS and a reducing agent β-mercaptoethanol (βME). PAGE of SDS treated protein allowed their separation based on their length in an easy & relatively accurate manner.

Zymography - Activity Staining

Zymographic analysis was carried out to estimate the approximate size of an enzyme sample, as per the ingenious Zymogram method devised by Heussen and Dowdle. [5]

- 12% SDS polyacrylamide gel copolymerized with gelatin (Separating gel)
- Tris - glycine - SDS buffer (Tank buffer)
- Electrophoretic apparatus
- 5X Gel loading buffer
- Protein standard marker
- Boiling water bath
- Gel running buffer
- Renaturation solution
- Development solution
- Staining solution
- Destaining solution

The active fractions of 10 fold TFF concentrated sample were subjected to electrophoretic characterization. The non denaturing SDS - PAGE protocol for vertical slab electrophoresis in 12% polyacrylamide gel according to the protocol described by Laemmli (1970) was used in the present investigation. The reagents used for the gel preparation and their concentrations are given in the **Table 1**

Table 1: Polyacrylamide Gel Preparation

Separating Portion (10%)		**Stacking Portion (4%)**	
Reagent	**Volume(mL)**	**Reagent**	**Volume(mL)**
D/W	3.1	D/W	2.5
1.5 M Tris - HCl (pH 8.8)	2.5	1.M Tris - HCl (pH 6.8)	0.625
30% Acrylamide	3.3	30% Acrylamide	0.67
20% SDS	0.05	20% SDS	0.025

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20% APS	0.05	20% APS	0.025
TEMED	0.005	TEMED	0.005

Sealing of Apparatus and Gel Casting

Polyacrylamide gel was cast with 12% resolving gel containing 0.1% gelatin. The glass plates (square plate and notch plate) with the spacers were cleaned and assembled in a casting stand and the apparatus was sealed with 1% agar solution. Proper sealing was checked by adding water between the two glass plates. The water was decanted off and its traces were removed by inserting Whatman filter paper between the two plates.

A 12% resolving gel solution was prepared by adding all the reagents in a beaker; excluding APS (Ammonium persulfate) and TEMED (N, N, N', N' Tetramethylene-1,2-diamine) which were added after degassing the solution. The solution was degassed and 30% Acrylamide - Bisacrylamide, 1.5 M Tris - HCl (pH 8.8), 10% SDS solution with TEMED and freshly prepared 10% APS were mixed. The TEMED and APS were added just before the gel was discharged between the gel plates. The resolving gel mixture was instantly poured into the cast in the gap between the two plates. The gel was allowed to polymerize for at least 45 minutes after pouring a layer of water over it.

The stacking gel components were taken in a beaker followed by gentle mixing of APS and TEMED. The contents were thoroughly stirred and poured above the resolving gel in the cast. The comb was instantly implanted between the glass plates and well - positioned in the gel as soon as possible. Trapping of air bubbles was avoided; the gel was allowed to polymerize at room temperature for at least 45 minutes After complete polymerization, the comb was removed carefully from the electrophoresis apparatus without tearing the edges of the wells.

Sample Preparation

B_3 extracted sample fraction was mixed with SDS - PAGE sample bufferresuspended in 5X Gel loading buffer in 1:4 ratio in Eppendorf tubes. The content of the tubes was mixed using a vortex mixer; tubes were heated at 100°C for 3 minutes. The sample was kept at 0˚C until it was applied to the gel and then separated on a 12% separating gel. It was loaded on to the gel with a standard SDS gel loading buffer as described. The standard marker used was prepared as per the manufacturer's specified procedure and was directly loaded on to the gel.

Gel Electrophoresis

The gel was taken in electrophoresis apparatus and the reservoir was filled with running buffer for SDS - PAGE. After polymerization, the gel was washed with Tris - glycine (tank buffer) buffer with the help of a syringe. Tris - glycine electrophoresis (tank) buffer was loaded on to the upper and lower tanks of the apparatus. The measured quantities i.e., 20 µL of 1:10 diluted semi - purified sample and 1:100 diluted Proteinase K was loaded was

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carefully loaded on to the gel with the help of micropipette using denaturing loading dye without boiling. The sample of commercial molecular weight marker was added in another well with a cover being placed above the gel chamber. The equipment was connected to an external electric supply unit; with the positive electrode affixed to the bottom of a buffer reservoir and negative at the top of the apparatus. The gel was run at an applied voltage of 100V in cold conditions of 4°C until the sample penetrated the resolving gel.

When the dye front reached the resolving gel, the potential was gradually increased to 120V. The gel was allowed to run until Bromophenol blue touched the bottom of the resolving gel. The run was ceased when dye front could reach 1.0 cm above the glass plate's lower extremity. After completion of the run, the gel was carefully separated from the cast, washed in running water and soaked in Triton X-100 for approximately 30 minutes. One half of the gel was added to 50 mM Sodium phosphate buffer solution at pH 7.0 for 1h and stained by dipping in Coomassie Brilliant blue G-250 for 1h.

The gel was then transferred to a destaining solution (10% glacial CH_3-COOH, 40% C_2H_5-OH and 50% water) until the clear bands appeared. The other half of the gel comprising of a duplicate set of samples was analyzed by silver staining method. All the samples together with the protein marker were run in duplicate. The protein band accountable for protease activity exhibited clearance on Gelatin - PAGE.

Silver Staining [6]

In the silver staining method, polyacrylamide gel wasimpregnated with soluble silver ions (Ag^+) and developed by a reductant. The amino acids notably the aromatic ones in the protein reduce silver nitrate to form yellowish brown to brown color complexes with metallic silver causing the protein to be visible.Post - electrophoresis, the gel was subjected to silver staining using Blum's protocol (1987) with slight modifications. The required stock solutions and reagents were prepared as given in the table below(**Table 2**).

Table 2: Gel Electrophoresis - Stock Solutions

Fixative Solution	**Quantity**
Methanol	100 mL
Acetic acid	24 mL
Formaldehyde	100 µL
Distilled water	76 mL
Wash Buffer	
Ethanol	73 mL

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Distilled water	127 mL
Sensitizer	
$Na_2S_2O_3$	0.04 g
Distilled water	200 mL
Developer	
Na_2CO_3	24 g
Formaldehyde	200 µL
$Na_2S_2O_3$ (0.02%)	8.0 mL
Distilled water	392 mL
Silver nitrate solution	
$AgNO_3$	0.4 g
Distilled water	200 mL
Stop Solution	
Methanol	100 mL
Acetic acid	24 mL
Distilled water	76 L

After electrophoresis, the SDS - PAGE gel was kept in a box and incubated in the fixative solution for 30 min. The gel was subsequently washed with CH_3-OH - H_2O mixture (wash solution) for 15 - 20 min. followed by 3 - 5 washes at 5-min. intervals so as to remove Acetic acid completely. Pre-treatment was done by incubating the gel in sensitizer (Sodium thiosulphate)solution for 60 sec. The gel was rinsed twice with distilled water at 60 sec. intervals for 2 minutes and then overlaid by chilled silver nitrate solution for 20 - 25 min. Proteins were allowed to react with silver ions and the solution was decanted after 20 min.

The gel was subsequently transferred to a developer solution and kept till the development of protein bands (usually the band develops within 10 minutes after addition of the development solution). The reaction was stopped when the development of bands got completed. After the appropriate staining procedure, the reaction was arrested by washing with Methanol-Water solution (stop solution).

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RESULTS & DISCUSSION

Morphological studies of the isolated bacterial cultures showed that the colony sizes varied from 1.0 - to 8.0 mm. Color variation of the bacterial suspension was from white, cream, yellow, orange to light brown. Their consistencies were found to be opaque and/ or translucent.Phylogenetic analysis of the potential bacterial isolates was carried out; PCR amplification of the 16S rRNA gene of the isolates B_2 and B_3 and their sequence analysis by BLAST tool showed that both the isolates belonged to the genus Bacillus. The two proteases producing bacteria B_2 and B_3 were identified as *Bacillus anthracis* and *Bacillus safensis* respectively.The optimum temperature for B_3 extracted sample was found to be 60°C and pH optima was found to be 7.0.The purified and concentrated sample was found to be highly stable over a broad range of pH from 5.0 - 9.0. The sample was also found to be thermostable upto the temperature of 60°C. [7,8,9]When the most proficient B_3sample under study was loaded and run on a gelatin-based polyacrylamide gel, clearance zone was observed at the band accountable for protease activity, apparently by the virtue of localized gelatin degradation. [10]

SDS PAGE Analysis

Protease activities of bands obtained in SDS PAGE were checked by performing Zymography of the semi - purified protease on SDS gel containing substrate gelatin. Results obtained by the analysis are shown in **Fig. 1 and Fig. 2.**

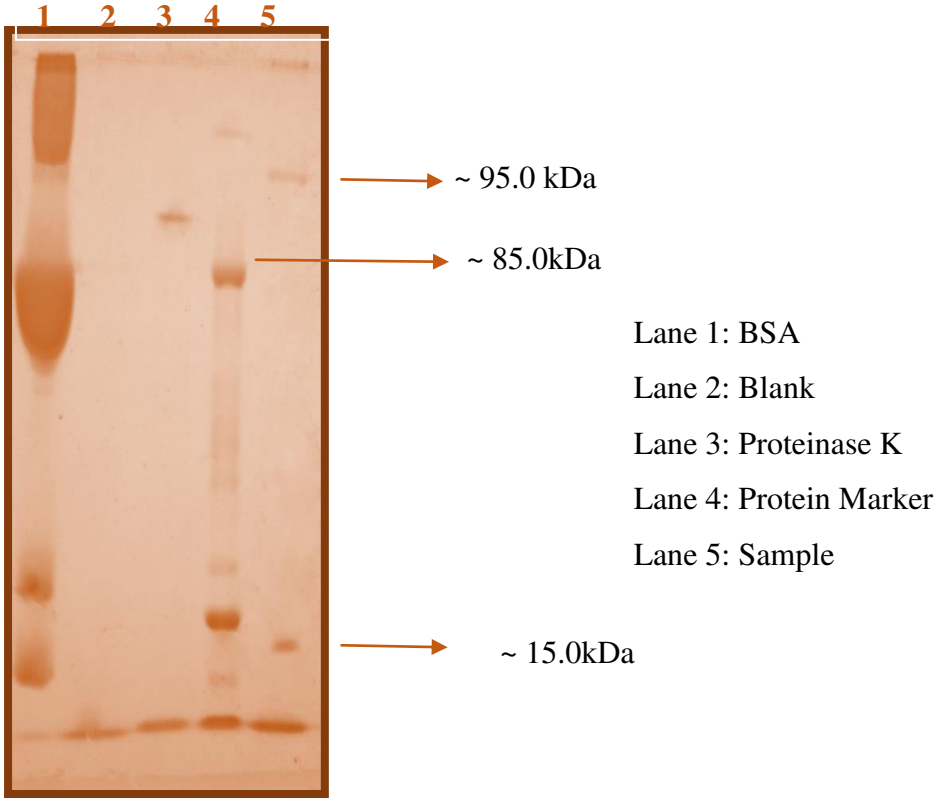

Fig. 1: Gel Electrophoretic Analysis

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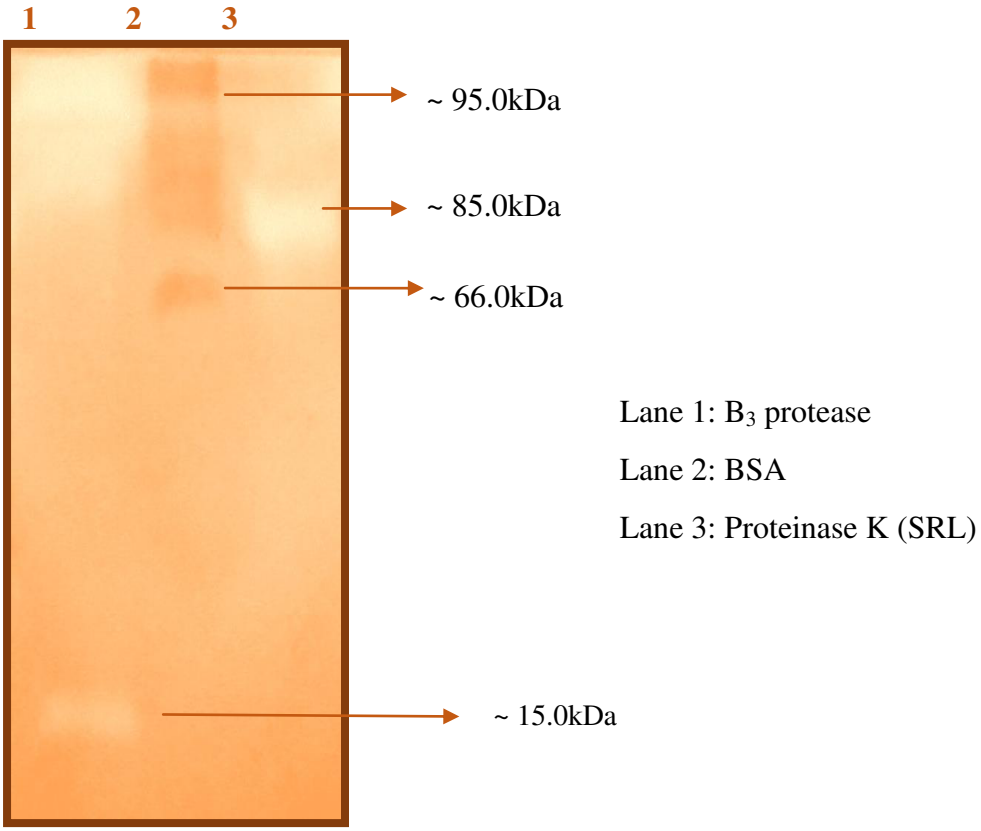

Fig. 2: Zymogram

From the figure, it can be seen that three clear protein bands were observed on gel post silver staining that corresponds to the bands obtained on SDS PAGE with molecular weight approximately 95 kDa and 15 kDa. Moreover, higher activity was observed at 95 kDa than at 15kDa when compared with bands obtained in the marker lane. Hence it was concluded that two types of proteases with molecular weight of 95 kDa & 15 kDa are present in the sample.

In literature, proteases with the molecular weights spanning as low as 16kDa to as high as 50 kDa have been are reported by Kaur (1998), [11]Gupta (2005) [12]Guangrong (2006), [13] Jaswal *et al*. (2007) [14]and Kim (2007) [15] respectively.

Mane and Bapat (2001) also obtained similar results for serine alkaline protease derived from *Bacillus subtilis* NCIM 2713. [16]Sorensen and co-workers (2002) have demonstrated a rapid and congruous method for silver staining of proteins on electroblotting membranes and intensification of the negatively stained or invisible proteins on silver-stained gels using SDS - PAGE technique to enable their visualization. [17]Dutta and Banerjee (2006) isolated extracellular proteases from *Pseudomonas sp*. with a low molecular weight of 14.4 kDa. [18]

The zymogram activity staining results as obtained by Almas Sadia *et al.* (2009) also indicated a clear zone of proteolytic activity formed owing to the degradation of gelatin; employed as a substrate in zymography. [19]Rama Krishna and co-workers (2011) purified protease from *Bacillus subtilis* KHS-1. They also performed activity staining of the protease by casein zymography. [20]Ishtiaq Ahmed *et al*. (2011) observed relatively lower molecular weight of

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27 kDa for *Bacillus subtilis* protease. [21]Williams (2012) characterized the neutral protease from *Bacillus subtilis* andreported the molecular mass of partially purified protease as 50 kDa using SDS PAGE. [22]Prihanto *et al*. (2016) extracted protease from *Bacillus subtilis* Ubt7 isolated from Terasi, an Indonesian fermented fish. After purification and characterization by gel electrophoresis, molecular weight of the neutral protease was recorded as 50 kDa. [23]

CONCLUSION

TFF concentrated sample was characterized by Zymogram SDS - PAGE. In the gelatin zymography, clear bands of protease activity were observed on gel which corresponds to bands obtained on SDS - PAGE. Zymogram analysis showed the presence of two types of proteases having molecular weight 95 kDa and 15 kDa in the sample.

ACKNOWLEDGEMENT

The first author is thankful to the University Grants Commission (UGC) for bestowing Teacher Fellowship Award under Faculty Development Program (FDP) Scheme. Laboratory & Infrastructural support from Principals, Smt. Chandibai Himathmal Mansukhani College & Guru Nanak Khalsa College is also gratefully acknowledged.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this article.

DATA AVAILABILITY

The data used to support the findings of this study are included within the article.

FUNDING STATEMENT

No funding is received for this research work.

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