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Screening and Production of Protease Enzyme from Marine Microorganism and Its Industrial Application

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Abstract: *Marine sediment samples were collected from the Gulf of Mannar, Mandapam coast to screen for protease producing microbes. Among the five isolates screened only two isolates showed maximum proteolytic activity with the zone of 21mm and 19mm respectively. Biochemical characterization of the isolates were performed and identified as strain P2 belonged to Bacillus subtilis and strain P5 belonged to Bacillus licheniformis. Both the strains have the ability to tolerate 7%Nacl concentration. The amount of protease produced was expressed in microgram of tyrosine released under standard assay conditions. The total protein content of crude enzyme extracts of Bacillus subtilis and Bacillus licheniformis were quantified which revealed 21.2mg/ml for strain P_2 and 22.4mg/ml of protein content was presented by strain P_5. The proteolytic bacteria gave an optimum performance were both strains exhibited the enzymes stable at P^H7. In the present study Bacillus subtilis showed a remarkable activity at 40ºC where as Bacillus licheniformis exhibited maximum activity at 50ºC. Studies pertaining to carbon sources starch and lactose were utilized by Bacillus subtilis and Bacillus licheniformis and maximum production was achieved. Among the different nitrogen sources tested yeast extract induced maximum proteolytic activity where as ammonium sulphate was found to be the best nitrogen sources for protease production. The crude enzyme was efficient to remove hair dye and blood stain by Bacillus subtilis and Bacillus licheniformis.*

Keywords: *Bacillus subtilis, Bacillus licheniformis, blood stain, detergent, hair dye, protease, stain removal, temperature.*

I. Introduction

Marine biotechnology is the science in which marine organisms were used in full or partially to make or to modify products, to improve plants or animals or to develop microorganisms for specific uses. [1]. There has been a tremendous interest from researchers to explore marine microorganisms as new source of antibacterial compounds as increasing resistance of pathogen to present antibiotics. Marine microorganisms were proven already to have many beneficial bioactivities such as production of industrial enzymes [2].

Bacteria were isolated and cultivated from all possible regions of the earth, on the basis of their habitat, diversity, ecological functions, degree of pathogenicity and biotechnological applications. 70% of the earth's surface is covered by oceans with rich microbial diversity. About 3.6×10^{29} microorganisms were found in marine environments, including subsurface and harbor [3].

Marine microbes were now being looked upon as a potential source of various compounds; pharmaceutical, nutritional supplements, agrochemicals, cosmetics and enzymes [4]. Proteases were a group of enzymes, whose catalytic function is to hydrolyses peptide bonds of proteins and break them down into polypeptides or free amino acids. They constitute 59% of the global market of industrial enzymes, which is expected to exceed $2.9 Billion by 2012. [5].

Proteolytic enzymes were degradative enzymes which catalyze the cleavage of peptide bonds in other proteins. Alkaline proteases, which are referring to proteolytic enzymes which work optimally in alkaline P^H are the main enzymes among proteases and constitute 60 to 65% of the global industrial enzyme market [6].

It has wide range of commercial usages in detergents, leather, food and pharmaceutical industries [7]. The genus "Bacillus" is an important source of industrial proteases and were probably the only genera being commercialized for alkaline protease production [8].

Many Bacillus species produce a variety of extracellular and intracellular proteases. Protease constitutes a large and complex group of enzymes that play an important nutritional and regulatory role in nature.

Proteases were (Physiologically) necessary for living organisms, and they are ubiquitous and found in a wide diversity of marine sources. Marine microbial enzymes have unique catalytic properties due to their distinct physiological and metabolic characteristics, efficient nutrient utilization in oligotrophic waters etc. and are also the source of novel biocatalysts like cold adapted enzymes which are economical in terms energy savings [9&10].

Microbial enzymes gained much attention due to their industrial application in clean, environmental-friendly and coast effective biotechnological processes. Proteases are also used in textile industry for removing the stiff and dull gum layer of sericine from the raw silk fiber leading to its brightness and softness. Alkaline proteases an interesting role in the decomposition of gelatinous coating of X-ray films from which silver was recovered [11].

In the present study it was based on screening and production of protease enzyme from marine microorganism and its industrial applications from Gulf of Mannar as it is not being well documented. Hence the present study will be carried out to screen and produce protease enzyme by venturing into marine ecosystem such as Gulf of Mannar Mandapam (coast) and their industrial applications being attracted with great attention.

II. Material And Methods

2.1. Isolation Of Marine Microorganisms From Marine Sediments

Marine sediment samples were collected from the Gulf of Mannar, Mandapam coast to screen for protease producing microbes. The collection was performed in plastic containers and transferred to laboratory at room temperature and the process of isolation was initiated immediately.

2.2. Screening for Protease Enzyme

The isolated colonies were screened for protease production using skim milk agar medium. All the isolates were streaked on to skim milk agar plates and the plates were incubated for 48 h at room temperature. The clear zones around the colonies were evaluated as protease producers.

2.3. Characterization Of The Effective Protease Producing Isolates

The bacterial isolates with prominent zone of clearance and showing efficient enzyme production were processed for the determination of colony morphology, Gram staining, biochemical tests and enzyme profiles and then identified in accordance with the Bergey's Manual of Determinative Bacteriology.

2.4. Identification And Characterization Of Isolated Organisms

The protease producing marine microorganisms were identified based on morphological and biochemical characterization methods.

2.5. Production Of Protease Enzyme

For enzyme production media consisting of Casein – 2.0%, Dextroses– 1.0%, Peptone - 1.0%, KH_2PO_4 - 2.0%, $NaCl_2$ – 0.2%, $CaCl_2$ – 0.002% at P^H 7.0 was used. Inoculums (OD 660 nm) were developed by growing the isolate in nutrient broth for 24 h. For production of enzyme, 1.0% inoculums was added to 50 ml production medium in 250 ml conical flasks and then incubated at 37℃ for 3 – 4 days. Sample withdrawn at specific time intervals were centrifuged at 10,000 rpm for 10 min and the supernatant has been used as enzyme source for assay.

2.6. Quantitative Estimation Of Protein

The quantitative estimation of protein was determined by the method Lowry's [12] using bovine serum albumin as the standard.

2.6.1. Determination Of Molecular Weight Of Protein In Sds -Page

The molecular weight of the crude enzyme was determined by sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) [13].

2.7. Protease Enzyme Assay

Proteolytic activity was carried out according to casein – pholine method culture media was centrifuged at 7200 rpm for 10 min and supernatant was used as enzyme source. However, 1% Casein (in 0.1 M phosphate buffer and P^H 7.0) was used as substrate. 1 ml each of enzyme and substrate was incubated at 50℃ for 60 min. The reaction was terminated by adding 3 ml of Trichloroacetic acid (TCA). One unit of protease activity was defined as the increase of 0.1 unit optical density at 1 h incubation period. It was then centrifuged at 5000 rpm for 15 min. From this, 0.5 M of supernatant was taken, to this 2.5 ml of 0.5 M sodium carbonate was added, mixed well and incubated for 20 min. Then it was added with 0.5 ml of folin phenol reagent and the absorbance was read at 660 nm using spectrophotometer. The amount of protease produced was estimated and expressed in microgram of tyrosine released under standard assay conditions. Based on the tyrosine released the protease activity was expressed in microgram of tyrosine released by 1 ml of enzyme in 30 minutes at 30℃ on tyrosine equivalent.

2.8. Optimization Parameters For Protease Enzyme Production

2.8.1. Effect of P^h on Enzyme Activity

To optimize the P^H of enzyme activity, 1 ml of enzyme solution was reacted with 5 ml of casein solution [1% (W/V)] in various P^H values (4, 5, 6, 7, 8, 9, and 10) at 37℃ for 30 min and the enzyme activity was assayed.

2.8.1.1. Effect of Temperature on Enzyme Activity

The influence of different temperature on proteolytic activity of the crude enzyme was determined by holding the reaction mixture at various temperatures (30ºC, 40ºC, 50ºC, 60ºC, and 70℃) for 30 min during standard enzymes assay.

2.8.1.2. Effect Of Carbon Sources On Enzyme Production

To find the optimum carbon source for enzyme production, five carbon source (1%) (Starch, Glucose, Sucrose, Lactose and Xylose) were supplemented to nutrient broth the organism was inoculated and incubated for 48 hr at 70℃ and the enzyme activity was assayed in the culture supernatant.

2.8.1.3. Effect of Nitrogen Sources On Enzyme Production

To optimize the nitrogen source for enzyme production, five different nitrogen source (1%) (Yeast extract, Gelatin, Casein, Urea and Ammonium chloride) were added to nutrient broth and the organism was inoculated and incubated for 48 hr. The enzyme activity was assay in the culture supernatant.

2.9. Application of Protease Enzyme: Wash Performance Test

Stability of the protease in commercial detergents were tested by incubating measured quantity of the enzyme (500µl) with the solutions of the different commercial detergent concentration of 7 mg/ml (to simulate washing conditions) for 1h. The detergents tested were Ariel, Tide (Procter and Gamble Ltd.), Rin, Surfexcel (Hindustan Lever Ltd.), and Henko (SPIC India Ltd.), which are widely used in India. Suitable aliquots were withdrawn at different time intervals (at15, 30 and 60 min), for 1h, and there sidual activity was measured by standard assay procedure and compared with the control (incubated under similar conditions, without any detergent) and the relative activity was expressed in % taking the value given by controlas100%. Application of protease as a detergent additive was studied on white cotton cloth pieces (5X5cm) stained with human blood, coffee and ink. The stained cloth pieces were taken in separate flasks. The following sets were prepared:

1. Flask with distilled water(100 ml) +stained cloth **(Cloth stained with blood, coffee and ink)**
2. Flask with distilled water(100ml)+1mldetergent (7 mg/ml)
3. Flask with distilled water (100ml) + 1ml detergent (7 mg/ml) + 2ml enzyme solution.

III. Results

3.1. Isolation and Screening Of Protease Producing Microorganisms

In the present study, different marine sediment samples were collected from Gulf of Mannar, Mandapam coast. The samples were serially diluted, spread plated and incubated at 37^{o}C for 24 hours.

About five dominant morphologically distinct colonies were selected and pure cultured by repeated streaking on the nutrient agar plates. The isolated five bacterial strains were screened for protease producing ability on skim milk agar. The zone formation around the bacterial growth was identified as the positive protease producers which may be due to hydrolysis of casein. Among the five isolates screened, only two isolates showed maximum proteolytic activity with a zone of about 21 mm and 19 mm whereas the other three isolates exhibited poor proteolytic activity. The results were noted in (**Plate No: 1).** Therefore these efficient protease producing strains P2 and P5 were selected for further experimental studies and biochemical tests.

3.2. Identification Of The Efficient Marine Protease Producing Organisms

Morphological and biochemical characterization were performed in accordance was Bergey's manual of determinative bacteriology. [14]. It was identified that strain P2 belonged to Bacillus subtilis and strain P5 belonged to Bacillus licheniformis. The results were depicted in (**Table: 1 &2, Plate No: 2 &3)**.

3.3. Salt Tolerance Of The Isolates

The salt tolerance of the isolates was studied at various NaCl concentrations such as 3% NaCl, 4%NaCl, 5%NaCl, 6%NaCl, and 7% NaCl. The results were shown in **(Plate No: 4)**. It was found that both the tested strains have the ability to tolerate up to 7% NaCl concentration.

3.4. Protease Enzyme Assay

Crude enzyme extract was studied for protein degrading activity and the amount of amino acid produced was measured by using Folin phenol reagent and the absorbance was read at 660 nm using colorimeter. The amount of protease produced was estimated and expressed in microgram of tyrosine released under standard assay conditions. Based on the tyrosine released the protease activity was expressed in microgram of tyrosine released by 1ml of enzyme in 30 minutes at 30˚C on tyrosine equivalent. The result was exhibited in **(Plate No: 5).**

3.5. Determination Of Total Protein Content

The total protein content of crude enzyme extracts of marine microorganisms such as Bacillus subtilis and Bacillus licheniformis were quantified by Lowry et al., method with BSA standard. [12]. It was found out that strain P_2 (Bacillus subtilis) revealed 21.2mg/ml, whereas strainp5 (Bacillus licheniformis) exhibited a remarkable increase of protein content of 22.4mg/ml. The results were presented in **(Table: 3)**.

3.6. Determination Of Molecular Weight By Sds - Page

The molecular weight of purified enzyme was determined by SDS - PAGE. The crude protease enzyme was concentrated by concentrated by centrifugation and approximately 90-100% of proteolytic activity was observed. **(Plate No: 6).**

3.7. Effect of P^h On Protease Enzyme Activity

PH played an important role in the enzyme production. In the present study the pH optima of protease activity was studied at different pH ranging from 4 to 10. The highest protease production was observed in P^H 7 for P2 with 0.176 u/ml and minimum production was observed in pH 10 (0.121 U/ml) whereas an enhanced protease production was observed in pH 8 for P5 (0.22 U/ml). Below and above that pH the enzyme activity was found to be decreased. The obtained results were presented in **(Fig: 1)**.

3.8. Effect of Temperatureon Protease Enzyme Activity

Temperature also played an important role in activating and inactivation of enzymes. In the present study, the effect of temperature on protease production was studied with various temperatures ranging from 30 - 70℃. The protease activity is relatively stable in the temperature range 40℃ for P2 (0.236 U/ml) and temperature range 50℃ for P5 (0.214 U/ml).The enzyme activity was found to be decreased above and below those temperatures for both strains. The present investigation showed that Bacillus subtilis and Bacillus licheniformis produced maximum protease at 40ºC and 50ºC. The obtained results were noted in **(Fig: 2)**.

3.8.1. Effect of Carbon Sources On Protease Activity

The effect of different carbon sources viz. Sucrose, Starch, Mannose, Fructose, Dextrose, and Lactose on enzyme activity was studied. Maximum protease production was observed in P2 when Lactose (0.275 U/ml) was supplemented as carbon source whereas an enhanced production was observed in Starch (0.198 U/ml) for P5. The least enzyme activity was observed in starch (0.159 U/ml) and dextrose (0.11 U/ml) for strains P2 and P5. The results were presented in **(Fig: 3)**.

3.8.2. Effect of Nitrogen Sources On Protease Activity

In the present investigation, Yeast extract, Urea, gelatin, casein and ammonium chloride were added in the medium for determining protease activity. Among the different nitrogen sources tested maximum protease production was observed at Yeast extract with 0.215 U/ml for strain P2 and Urea for strain P5(0.204 U/ml).

The addition of other nitrogen sources caused a drastic reduction in enzyme activity. The addition of urea (0.1375 U/ml) and gelatin (0.126 U/ml) in the medium decreased the enzyme production in P2 and P5. These results were noted in **(Fig: 4).**

3.9. Application of The Protease Enzymes In Stain Removal:

In order to find out the dye removal efficacy of the strains, a white cotton cloth (5 x 5 cm) was stained with hair dye (godrej) and dried in hot air oven for one hour. Then the stained cloth was incubated at 50^{o}C in enzyme broth for different time intervals. The enzyme was also able to remove hair dye from the test fabric cloth after 2 hours at 50^{o}C for Bacillus licheniformis and 2 hours and 45 minutes for Bacillus subtilis. These results were noted in **(Plate No:7).**

The enzyme was also efficient to remove blood stains within 30 minutes for Bacillus licheniformis at 50^{o}C whereas Bacillus subtilis completely remove blood stains in 45 minutes at 50^{o}C. It was seen that protease produced by both Bacillus sp had high capability of removing the dye and blood stains from fabric cloth, which indicated its potential in detergent industries. The obtained results were noted in **(Plate No: 8)**.

From the present study it is revealed that the protease enzyme produced by the marine microorganisms showed a positive result in the removal of dye and blood stains from the cloth. Therefore this enzyme was used in the industrial applications in pilot scale.

IV. Figures And Tables

FIG: 1

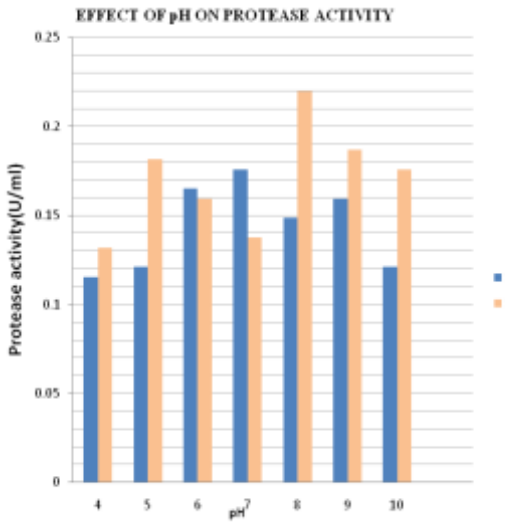


FIG: 2

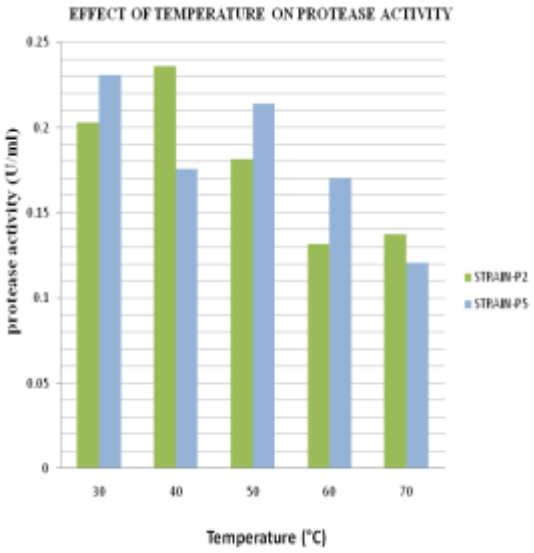


FIG:3

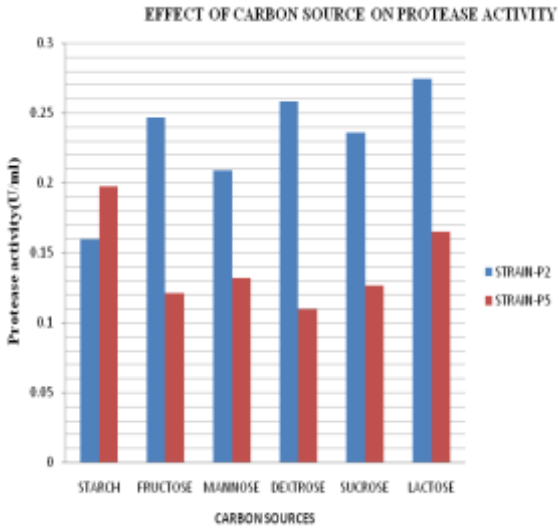


FIG: 4

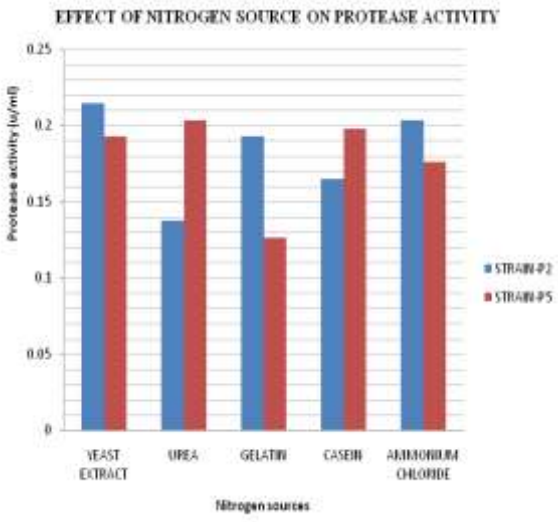


Table 1: Morphological and Physiological Characterization of the Efficient Protease Producing Isolates

S. No	Tests	Bacillus subtilis sp	Bacillus licheniformis
1.	Colony morphology.	On nutrient agar, it produced creamy white, irregular undulate colonies.	Whitish to brown colour, dull rough, surface, irregular colonies
2.	Morphological characteristics. a) Gram staining b) Spore staining	Positive rod Endospore forming	Positive rods Present

Table 2: Biochemical Characterization of the Efficient Protease Producing Isolates

S. No	Biochemical tests	Bacillus subtilis sp	Bacillus licheniformis
1.	Indole production test	Negative	Negative
2.	Methyl red test	Negative	Negative
3.	Voges Proskauer test	Negative	Negative
4.	Citrate utilization test	Negative	Negative
5.	Triple sugar iron test	Alkaline slant	Alkaline slant
6.	Gelatin hydrolysis	Positive	Positive
7.	Nitrate reduction test	Positive	Positive
8.	Starch hydrolysis test	Positive	Positive
9.	Carbohydrate fermentation tests a) Lactose b) Sucrose c) Dextrose d) Mannitol	 Negative Positive Positive Positive	 Negative Positive Positive Positive

Table 3: Estimation Of Protein By Lowry's Method

S.NO	Volume of Working Standard (ML)	Concentration (µG)	Distilled Water (µL)	Reagent C (M/L)	Reagent D (M/L)	OD AT 660NM
Blank	-	-	1.0	5	0.5	0.00
S_1	0.2	40	0.8	5	0.5	0.22
S_2	0.4	80	0.6	5	0.5	0.27
S_3	0.6	120	0.4	5	0.5	0.40
S_4	0.8	160	0.2	5	0.5	0.53
S_5	1.0	200	-	5	0.5	0.60
P_1	1.0	200	0.5	5	0.5	0.76
P_2	1.0	200	0.9	5	0.5	0.71

Plate: 1

Plate:2

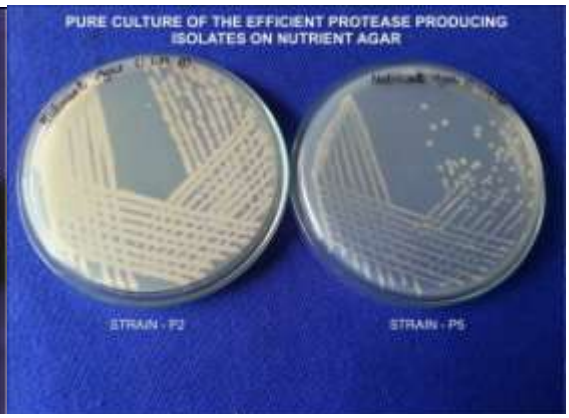


Plate: 3

Plate:4

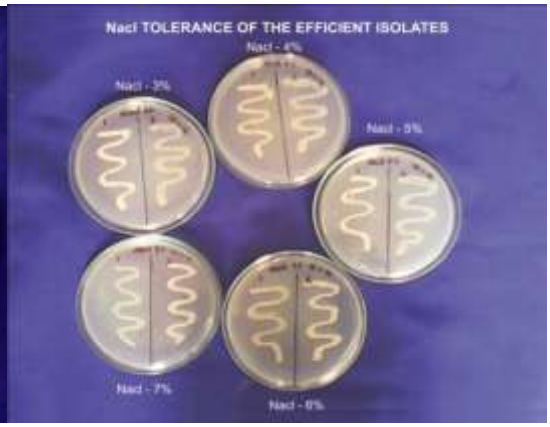


Plate: 5

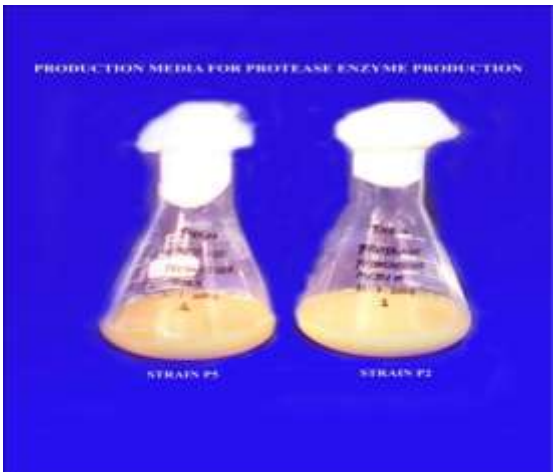


Plate:6

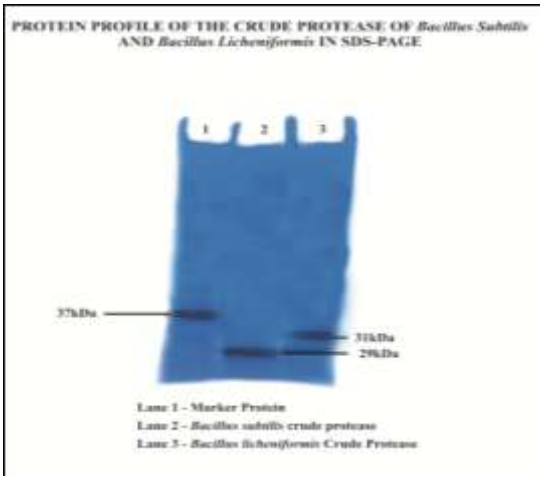
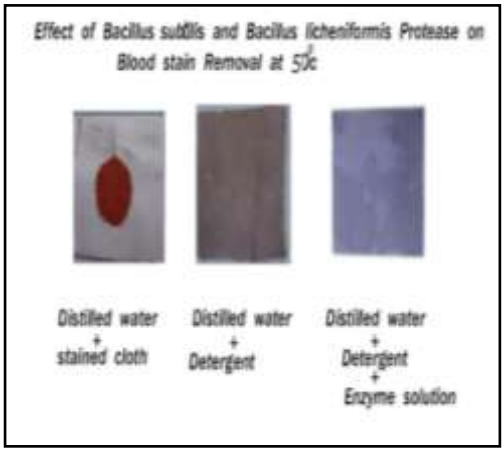


Plate: 7

Plate:8



V. Discussion

Proteases from microbial sources are preferred than the enzymes obtained from plant and animal sources since they possess almost all characteristics desired for their biotechnological applications [15].

Proteolytic bacteria are widespread in nature and are able to grow under various growth conditions, such as different temperatures, p^H and ionic strength [16].

The bacteria that exist in the marine samples represent a very important and diversified enzymatic potential. But sufficient information is not much available on their role in industrial and hence the present study was carried to isolate and characterize the potent protease positive bacteria from the marine sediment sample of Gulf of Mannar, Mandapam (coast).It has been reported that the production of extracellular proteases by different microorganisms can be strongly influenced by the culture conditions. So, it becomes necessary to understand the natural proteases and their catalytic potentiality under different conditions. [17].

In this experiment, 5 bacterial isolates were able to produce proteolytic enzymes. The results indicated that the five proteolytic bacteria and among two positive isolates was the dominant protease producer it was identified as Bacillus sp., Among the proteolytic marine bacteria isolated from sediment samples, gram positive rods were predominant. Formation of clear zone on skim milk around the isolated colonies was considered as indication of good growth with the ability of protease production, therefore, the largest zones producing strains were selected for further study.

In this study, strain p2 achieved the largest proteolytic zone with respect to the colony diameter (21mm) and strain p5 have the largest proteolytic zone with respect to the colony diameter (19mm) and its optimum growth estimated at higher alkaline range (3-7), therefore, this strain was chosen for further studies. Microbial proteases are produced from high yielding strains including species of bacillus sp., among these, Bacillus subtilis, Bacillus licheniformis is the most important group of bacteria that are involved in the enzyme industry and this bacterium is also known to produce proteolytic enzymes quite effectively [18].

Determination of molecular weight by SDS-PAGE for the separated fractions of the protease enzyme exhibited a predominant peak (band) of 29 kDa which indicate that protease have high activity and this lyse within the molecular weight range of standard marker between 14-160kDa protein marker.Our results are in total agreement with the work of [19&20] which emphasized that protease fractions of appeared as a single band just below 29 kDa isolated from B.species. It is concluded that B.licheniformis capable of producing proteolytic enzyme giving optimum performance and serves to be an ideal potential candidate for industrial applications.

The enzyme was considerably stable at pH 7 and 8 for both strains, but below and above that pH the enzyme stability gradually decreased. Similar results were reported by [21]. The present observations disagreed with the results of [17] in which they reported that Bacillus sp was highly active and stable at pH 9-10. [23] reported that optimum protease activity and stability was recorded at pH 9-9.5.

Temperature plays an important role in inactivation and activation of enzymes. Each enzyme has an optimum temperature for maximum enzyme activity. In the present study, Bacillus subtilis showed maximum activity at 40^{o}C.

Our results coincide with the work of [21] in which he demonstrated that at temperatures of 60 and 70^{o}C, the enzyme lost its activity rapidly where as the optimum temperature were found to be 40^{o}C. In our study, Bacillus licheniformis exhibited maximum activity at 50^{o}C.

The results of the present study agreed with the mentioned results of [24]. The addition of carbon sources in the medium influence the production of protease enzyme. Maximum production was observed in starch and lactose for B.subtilis and B.licheniformis. It is worthy to note that except starch and lactose, the addition of other carbon sources in the production medium led to the remarkable decreases in protease production. These results are in accordance with the finding of [25].

The nitrogen sources are the secondary energy sources for microorganisms which play an important role in the growth of the organisms and enzyme production. In the presence study, the extra addition of yeast extract to the medium induced the proteolytic activity. The other sources showed no remarkable increase or depressive effective on protease production. Similar work was carried out by [26].

They observed maximum production when malt extract was used as nitrogen source reported that ammonium sulphate was found to the most effective nitrogen source for protease production.In laundry detergent the use of enzymes such as proteases in very common about 50% of liquid detergent, 25% of powder detergent had almost all powder bleach additives now contain enzymes to help in breaking down of stains that are otherwise hard to remove with conventional surfactants alone. They work as scissors to cut off the stain physand piece by piece from the surface of the fabrics. [27].

The efficiency of B.subtilis and B.licheniformis proteases were tested for removing the stains and hair dye stain from test fabrics at 50°C with enzymes and 1% V/V of 15mg/ml detergents (surfexel). The enzyme was efficient to remove blood stain within 30minutes for B.licheniformis where as B.subtilis completely remove the stain within 45minutes.the enzyme was also able to remove hair dye and also showed computability with the detergents the protease enzymes were also used as an additive detergent to check the contribution of the enzymes in improving the washing performance of the detergents.

The supplementation of the enzyme preparation in surfexel could significant improving & cleansing performance towards proteinase stain (blood stain & hair dye). Our work coincides with the work of [28].

VI. Conclusion

Proteases are important enzymes obtained from marine microorganisms which synthesis bioactive compounds. The bacterial strains isolated from marine environment where identified as Bacillus subtilis and Bacillus licheniformis. Screening was performed and maximum proteolytic activity was revealed. The strain P_2 and P_5 have tolerated up to 7% NaCl concentration for enhanced production more over P_2 and P_5 exhibited excellent growth at 40^{0}c. Whereas optimization of P^{H} revealed the strain P_2 exhibited to increased protease production at $P^{H}7$ followed by strain P_5 revealed an optimum $P^{H}10$ for enhanced protease production. Different carbon sources tested starch and lactose was found suitable for protease production for strain P_2 and P_5 respectively. Similarly supplementation of nitrogen sources was found favorable for strain P_2 where as P_5 utilized urea as nitrogen source.

Molecular analysis of the crude enzyme showed prominent band with the molecular weight of 29Kda for strain P_2 and 31 Kda for strain P_5. The role of protease enzyme in wash performance using detergent was found to hydrolysed large protein molecules associated with hard stains. Hence its imperative to use the crude protease enzyme for stain removal.

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