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ORIGINAL ARTICLE

Bioprospecting of alkaline protease by a *Micrococcus luteus* MG603678 bacterium isolated from coastal environment

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ABSTRACT
The study aims at the characterization of purified alkaline protease Micrococcus luteus strain isolated from marine sediments collected at Tuticorin, Tamilnadu, Southeast coast of India.The16S rRNA sequencing and biochemical characterization exposed that the strain resembles Micrococcus luteus. The protease purification produced 2.5 fold purification with 2490 U/mg and 48.6% yield. The molecular weight of the enzyme was determined as 41.1kDa by SDS PAGE and exhibited activity and stability of pH (2-12) and temperature (20-80ºC). Metal activity of protease was studied which revealed highest activity with Mg^{2+} (90.2%), Ca^{2+} (85.3%) and reduced activity with metals such as Cu^{2+}, Fe^{2+}, $Zn^{2+}$and Co^{2+}. The partially purified alkaline protease was used to test various applications such as removal of hair from goat skin (activity at 10 h), breakdown of egg albumin (activity at 6 h), haemoglobin hydrolysis (activity at 12 h) wash performance of blood stain (9 h) and decomposition of gelatinous layer from used X-ray film (4 h). All the experiments have shown excellent results and hence it can be concluded that protease production from marine bacterium can be used in industrial and several biotechnological applications.

Keywords*: Alkaline protease; Micrococcus luteus; purification; dehairing; haemoglobin hydrolysis; decomposition of gelatinous layer.*

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INTRODUCTION

Proteases make the largest group of industrially significant enzymes hydrolyzing the peptide bonds linking proteins and amino acid residues. Proteases are ubiquitous in nature and can be obtained from animals, plants and microbes [1-3]. Proteases possess diverse potential applications in different industries such as laundry, pharmaceuticals, peptide synthesis and meat processing [4-7]. It could be noted that the largest contributor of microbial proteases is bacteria (40%) (8). The significant properties of bacterial proteases are dehairing [9] destaining [10-11] food processing [12], removing raw silk and silk fibers in textile industry [13] and silver recovery from devastated X-ray film [14-15].

Marine environments possess a diversity of promising bacteria due to the high amount of organic load as a result of anthropogenic activities particularly in tropical or temperate areas (16). The intricateness of the marine environmental conditions, may lead to diverse applications by marine microorganisms than terrestrial microorganisms, which boosted the marine microbial enzyme technology over the last few years and ended with high-quality products [17].

Bio prospecting of marine microorganisms have vast prospective for development and applications with industrial benefits however they are understudied. The present bio prospecting research deals with the screening and characterization of proteolytic enzymes, enzymatic action on diverse environmental conditions and its potential applications such as dehairing, breakdown of egg albumin, blood clot, blood stain removal and decomposition of raw gelatinous layer and release of silver from used X-ray film.

MATERIAL AND METHODS

Sample Collection

Sediment sample has been collected from Tuticorin in the South East coast of India. The sediments were placed in sterile Zip lock bags. The collected samples were placed in Insulated boxes and transferred to the laboratory for analysis.

Isolation of protease producing bacteria from marine sediments

Sample processing

The collected sediment samples were consecutively diluted and plated on skim milk agar medium then incubated at room temperature for 48hrs. Colonies showing clear zones were scored as positive. The selected strains were further grown individually in a minimal broth medium followed the by the slightly modified method of [18] comprising of casein 10.0 (w/w), K_2HPO_4 0.5(W/W), KH_2PO_4 0.5(w/w) and pH 7.0. The cultured pellets were incubated at room temperature for 72 hr. The cultured pellets were then centrifuged at the speed of 10,000 rpm at 4 ºC for 10 min, the cell free supernatant was collected and used as enzyme source.

Identification of protease producing bacteria

The Strain CASMM1 was characterized by physical (Gram staining) and biochemical methods according to the laboratory manual in general microbiology (19). The CASMM1 strain was identified by the method of 16S rRNA gene sequence. The bacterial genomic DNA was isolated by phenol chloroform method and 16S rRNA was amplified using the universal 8F and 1544R bacteria-specific primers that were designed to amplify a 1401 bp segment of the 16S rRNA. The amplified DNA fragments were sequenced and submitted to the Gen Bank database [20].

Qualitative Assay

Gelatin agar plates were prepared and 0.5cm diameter wells were made on the gelatin agar plates. The filtrate enzymes were added in the wells and kept incubated at 37 ºC. After 24 hrs, the plates were swamped by mercuric chloride solution (15 g in 20 ml of 6 N HCl) and clear zone formation indicated protease activity [21-22].

Quantitative Assay

For the quantitative analysis, the method described by (23) was followed, 0.5 % (w/v) casein solution was mixed with the enzyme solution (dissolved in 50 mM citrate buffer with pH of 7.6) and incubated for 60 mins at 37 °C. A unit volume (1ml) of Trichlororacetic acid [10 % (w/v)] was added and kept at room temperature for 10 mins which was later centrifuged at 10,000 rpm for 10mins. The 0.5 ml aliquots supernatants were mixed with 2.5 ml of 0.5 M Na_2CO_3 and 0.75 ml of Folin-Ciocalteu's phenol reagent: water (1:3 v/v) and kept incubated in a dark place at room temperature for 30 minutes. The measurement for optical density was determined at 650 nm by UV-Visible Spectrophotometer (Model No. Shimadzu UV-1800) and compared against the Tyrosine standard curve. The controls and blanks were carried at zero time without the supplemental enzymes.

Purification of protease enzyme

CASMM1 culture was centrifuged at 15000 rpm for 30mins and the 48 h grown cells were collected and precipitated to 80 % by the addition of $(NH_4)2SO_4$. The fractions were dialyzed with Tris-HCl buffer (pH-9) and the enzyme activity was determined under standard assay conditions [24].

SDS PAGE test

The enzyme denaturation was carried out at 100ºC and loaded on 10% (w/v) SDS–PAGE [25]. Examinations of protein band were done by Coomassie a Blue R-250 stain and molecular weight marker was used to calculate the molecular weight.

Biochemical categorization

Effect of temperature and pH on the protease activity and stability

The activity and stability of alkaline protease was performed at various ranges of temperature (20-80 °C) and pH (pH 2-12) following the method of [26].

Effect of metal ions and supplements on the protease

The basal medium was prepared with the supplements of l.0 % of glucose, lactose, fructose, maltose, sucrose, galactose and starch each to investigate the effects of carbon sources [27].

To examine the results of nitrogen sources, the basal medium supplements were altered by 1.0% of different nitrogen sources (peptone, yeast extract, triptone, casein, ammonium sulfate, ammonium nitrate, ammonium chloride and urea).

Protease activity on heavy metals was performed by inoculating the media with 10 mM of Cu^{2+}, Co^{2+}, Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+}.

Biotechnological applications

Removal of hair from goat skin

The goat skin was cut to pieces and incubated with the crude protease (2 U/ml) in 50 Mm Tris-HCL (pH 8) at 50 ºC. The skin was incubated for 10 h and the hair removal was observed [21, 28].

Digestion of Natural proteins

The crude enzyme (10 U) was incubated with egg albumin and blood clots in 20 mM Tris–HCl (pH 8) at 37 ºC. The substrates condition was observed at different time incubation [21, 29].

Wash performance assay

A piece of white material was soaked in goat blood and dried. The cloth stained with blood was soaked in buffered formaldehyde (2 %) for 30 min and excess amount of formaldehyde was removed using water wash. The equal sizedblood cloth chops were soaked with the purified protease (5 U) with different incubation time at 45-50 ºC. After incubation time, each piece of cloth was rinsed with water for 3 min and then dried. The same procedure was done for the control except incubation with the protease enzyme solution [21, 29].

Recovery of silver from waste X-ray photographic film

The waste X-ray films were made into pieces, cleaned with Milli Q water and swabbed with ethanol soaked cotton. After, the film was desiccated in an hat air oven at 40 °C for 30 min also treated with 15 ml of purified alkaline protease (1000 U/ml) at pH 9.0 and 40 °C, kept in Orbital shaker.The dried film was checked for decomposition of gelatinous layer [30].

Statistical analysis

All the experiments were done in triplicates; the mean values with standard deviation errors were processed and calculated by using the software version (SPSS, 16).

RESULTS AND DISCUSSION

Isolation, identification and screening of alkaline protease producing marine bacteria

A total of 36 bacterial strains were isolated from the sediments of Tuticorin coast, southeast coast of India. All the strains were tested for protein hydrolysis on skim milkagar plates and among them only three exhibited highest protease activity. The strains labeled as, CASMM1, CASPRS2and CASPRS3 was inoculated on gelatin agar and a zone of clearance around the well pointed out higher protease activity. Among the selected three strains, CASMM1 displayed significantly higher protease activity (740 U/ml) (fig.1). The cell-free supernatant of the bacterium CASMM1 was utilized for further studies. During the gram staining test, CASMM1 isolate was observed to be gram-positive and this strain was subjected to further biochemical tests. The strain showed positive activity for catalase and oxidase, while the other results such as indole, methyl red, VP, Simmon citrate showed negative activity. The strain showed a good protease activity, CASMM1 was selected for the further characterization, and the nucleotide sequence of 16S rRNA gene was submitted in the GenBank database. The phylogenetic analysis of the strain was compared with other closely related species in a dendogram. The study confirmed that the isolated strain belongs to the species of ***Micrococcus luteus*** (fig.2) and accession number MG603678 was obtained for the same.

Screening and characterization of alkaline protease

Protease purification steps are summarized (Table 1). After dialysis, the enzyme resulted in 2.5-fold increase, which exhibited 2490 U/mg specific activity of protein with 48.6% yield. A former study yielded1.9 fold increase activity and 1960 U/mg and 44.3 % yield (21) which were lesser than the present study. Renganathan *et al*., [21] demonstrated partially purified alkaline protease enzyme, with molecular weight of 36 kDa. However, a former study yielded a higher result at 40 kDa [22].The present study estimated molecular mass of the purified protease as 41.7 kDa (fig 3) which was similar to the results of [22].

Effect of pH and temperatureon enzyme activity and stability on protease

Effect of pH with protease production was observed at a pH range of 2-12. The highest enzyme production was achieved at 8pH (1005 ± 50.3 U/ml/min) which confirms that the produced enzyme was alkaline in nature. Rao *et al.*, [31] stated that most of the large the commercial microbial proteases exhibit maximum activity in the alkaline pH range of 8.0-12.0. Peak enzyme activity was noticed in pH 8.0 at 40 ºC, which is ideal for alkaline proteases [32]. The purified protease from M. *luteus* was active throughout the temperature (20-80 ºC) and pH (2-12) ranges used in this study. Maximum enzyme stability was achieved in pH 8.0 at 40 ºC however the stability of the enzyme was noticed from 20-80 ºC and pH (2-12) ranges used in this study (fig.4). The experiments were performed as triplicates. Mean standard deviation was calculated (Table 2 and 3).

Effects of metal ions and protease inhibitors on protease activity

The metal ions Mg^{2+} and Ca^{2+}significantly increased activities of the protease (90.2%, 85.3% respectively) while with the other metals (Cu^{2+}- 62.7% , Fe^{2+}- 64.9% , Zn^{2+}-59.9% , Co^{2+}- 50.6%) the activity reduced

considerably (fig.5a). Earlier works also support our findings where increased activity of alkaline protease by *Bacillus* sp. and *Bacillus megaterium* RRM2 upon addition of Ca^{2+} [13] and decreased activity by Hg^{2+}, Zn^{2+} and Co^{2+} (33-34) were noted.

Effect of various Carbon source, Nitrogen source on the purified protease activity

The protease synthesis of is greatly influenced by compositions nitrogen and carbon in the culture medium [35-36]. The present result showed that the media containing 1.0 % glucose as a carbon source supported highest protease production compared to the other carbon sources. Among the various nitrogen sources investigated, casein showed the highest level of protease production (fig.5b and c). The amount of protease production was noticed in the supplemented with Lactose (160 U/ml), Glucose (210 U/ml), Maltose (130 U/ml), Starch (100 U/ml), Fructose (20 U/ml), Sucrose (40 U/ml), Galactose (150 U/ml). The production status for nitrogen was in the order Tryptone (100.0 %) Casein (150.0 %), Peptone (90.0 %), Yeast extract (50.0 %), Urea (10.0 %), Ammonium chloride (2.5 %), Ammonium nitrate (1.5 %). These observed results are in comparable with earlier observation wherein diverse sugars supplemented [37].

Dehairing property of protease tested with goat skin

Similarly, the results of dehairing assay showed that, the enzymes produced by the *Micrococcus luteus* isolate expressed prominent dehairing activity after 10 hr (fig.6.a1, a2, a3) incubation which yielded a better result than a former study with *Bacillus pumilus* [38] and *Bacillus* sp. [28]. The results of the experiments illustrated that complete removal of goat skin with clear smooth white colored surface without any damages.

Protease exhibited better hair removing activity when compared to chemical dehairing method as the results of former studies indicate alkaline proteases as prospective tools for dehairing of hide in the leather industries with no perilous effects on the environment [27, 39]. In the present study, an alkaline protease was purified and this enzyme showed considerable activity against keratin as evidenced by its activity on goat skin. The major fraction of goat skin is keratin and it is present in hair, skin, nails and horns. Hence, this protease could be used to convert keratin into an eco-friendly product [40].

Digestion of Natural proteins

Breakdown of egg albumin

Egg albumin activity exhibited breakdown at 6 hours (Fig.6. b1and b2), which was comparatively faster than previous study [29] which took 14 hours for albumin breakdown.

Dissolving of blood clot

Blood clot removal test was performed to study the amount of time taken by the enzyme to remove the blood clot and in the current study, blood clot was removed within 12hrs (fig.6.c1 and c2) while a former study by [29] yielded 14 hrs for clot removal).

Wash performance assay

The alkaline protease obtained from *Micrococccus luteus* was able to remove blood stain from cotton cloth at room temperature within 9 h (fig.6.d1, d2, d3 and d4). Some of the earlier investigations, the researchers have examined the effectiveness of alkaline proteases in the blood stain removal of from cotton fabrics [2, 41]. The addition of proteases to the detergents cleans the stain by removing the protein content in it thereby consuming the substances active on the surface, thus reducing the pollution load caused due to these blood stains [41]. The remarkable stability of protease towards detergent and oxidizing agents appears to be a striking feature from the application stand point [9]. Previous studies have also reported the worth of alkaline proteases from alkaliphillic bacteria like *Pseudomonas* sp. *Spilosona* sp, and *Bacillus* sp. for the removal of blood stains from cotton clothing with or without the utilization of detergents [42-44]. The bacterial protease efficiency to digest different natural proteins such as blood clot, albumin, collagen and fibrin has been reported in the previous studies [29, 43, 45]. This ability to act in the presence of solvents and detergents could be exploited for other purposes.

Decomposition of gelatinous layer from used X-ray film

X-ray film contains a massive content of silver as compared with other variety of film; hence, Ag can be retrieved from exhausted X-ray film for reprocess. An estimate of about 18–20 % of the world silver requirement is satisfied by recycling these photographic/radiographic wastes [46]. The silver ions are bounded with gelatin in the emulsion layer, since it is feasible to breakdown the linkage and separate the silver by using proteolysis enzymes. In the present observation, the purified protease broke down the gelatinous layer on the X-ray film within 4 h of incubation (Fig.6 e1 and e2). This result indicated that, the alkaline protease produced from *M. luteus* has facilitated the recovery of silver from X-ray films. In an earlier study Al-Abdalall and Al-Khaldi, [14] recovered silver from alkaline protease of *B. subtilis* sub sp. Hakim *et al.*, [30] observed the capable exclusion of silver from partially purified protease *synthesized* by *B. subtilis.*

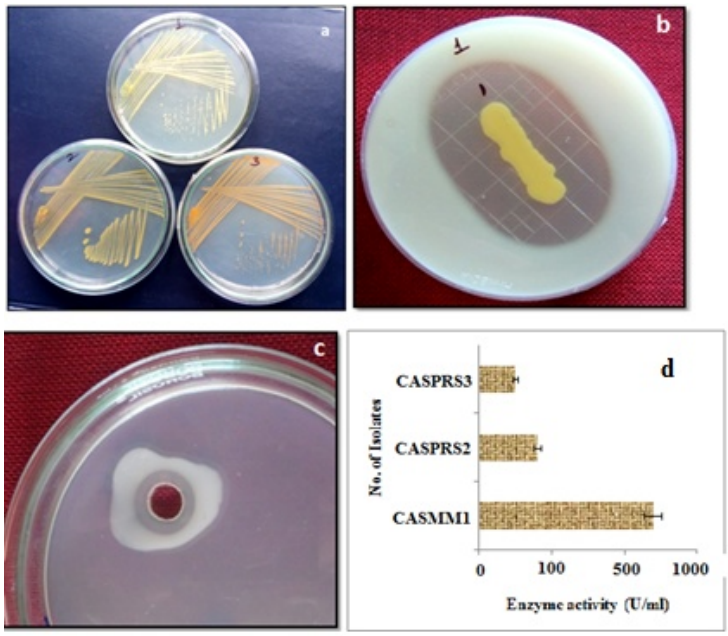

Fig.1. (a) Isolation of bacteria from sediment sample, (b) confirmation test on Skim milk agar colony shows the clear zone, (c) Qualitative assay of isolate CASMM1 in gelatin plate assay (The culture supernatant of the strains containing the extracellular protease was analyzed, Zone of clearance indicates the hydrolysis of gelatin by the enzyme), (d) Quantitative assay of Protease production by different isolate

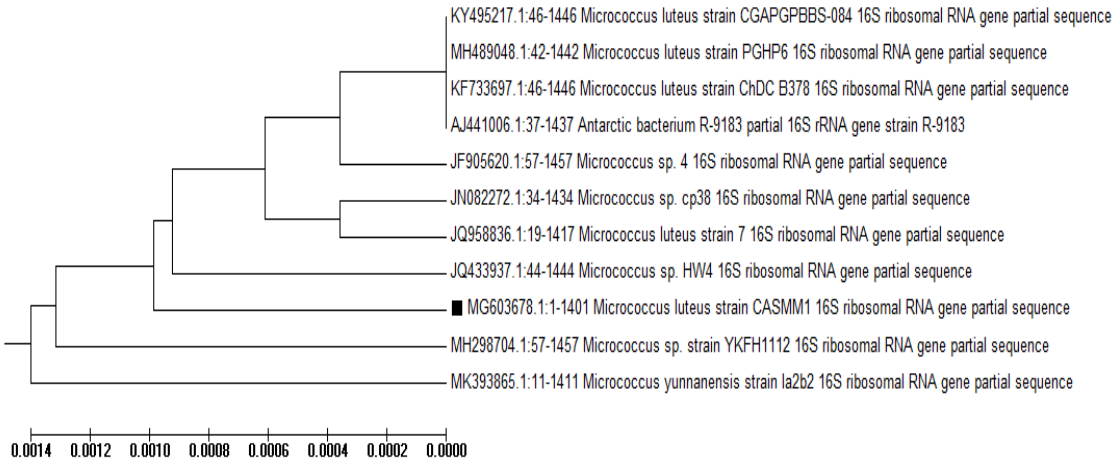

Fig.2. Neighbour joining tree of *Micrococcus luteus* (CASMM1) MG603678 based on partial 16S rRNA gene sequences

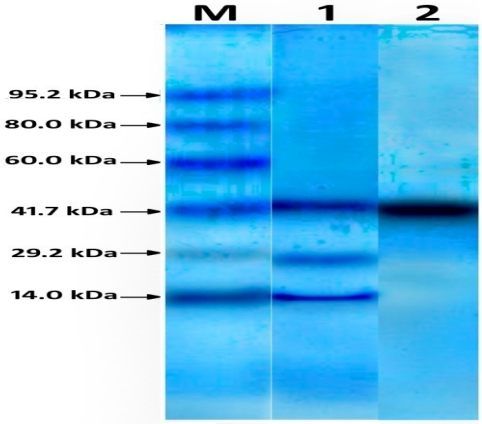

Fig.3. SDS PAGE analysis of protease enzyme from *Micrococcus luteus* M-Standard protein marker, 1.Crude protease 2. Purified protease

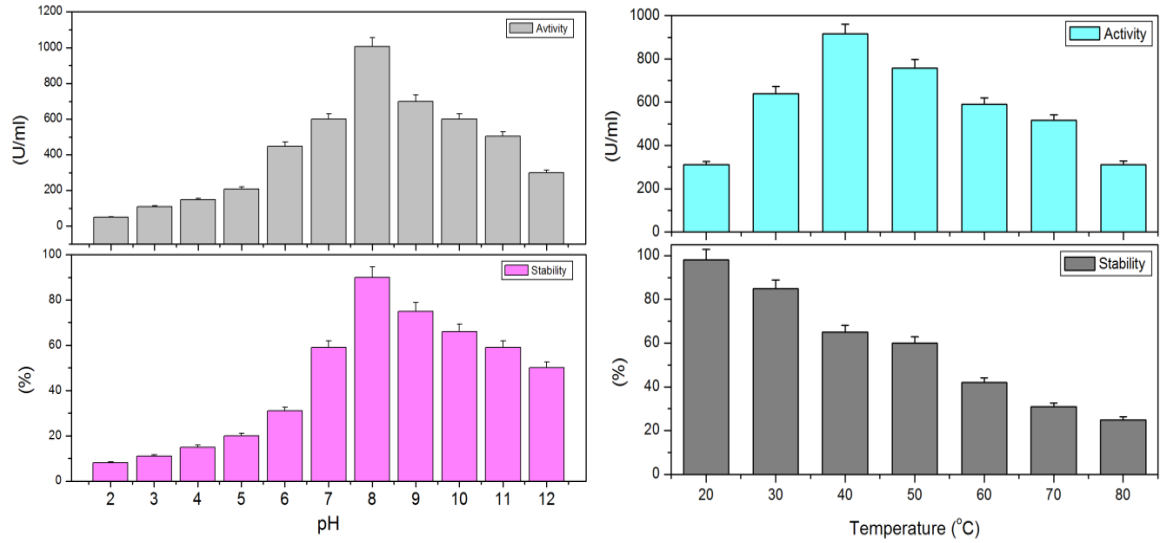

Fig.4. Effect of pH on enzyme activity, stability. The pH profile was determined in different buffers of varying pH (2.0-12.0) at 37°C. The temperature activity, stability of the protease enzyme was tested by pre-incubating at different temperatures for 1 h from 20° C to 80° C.

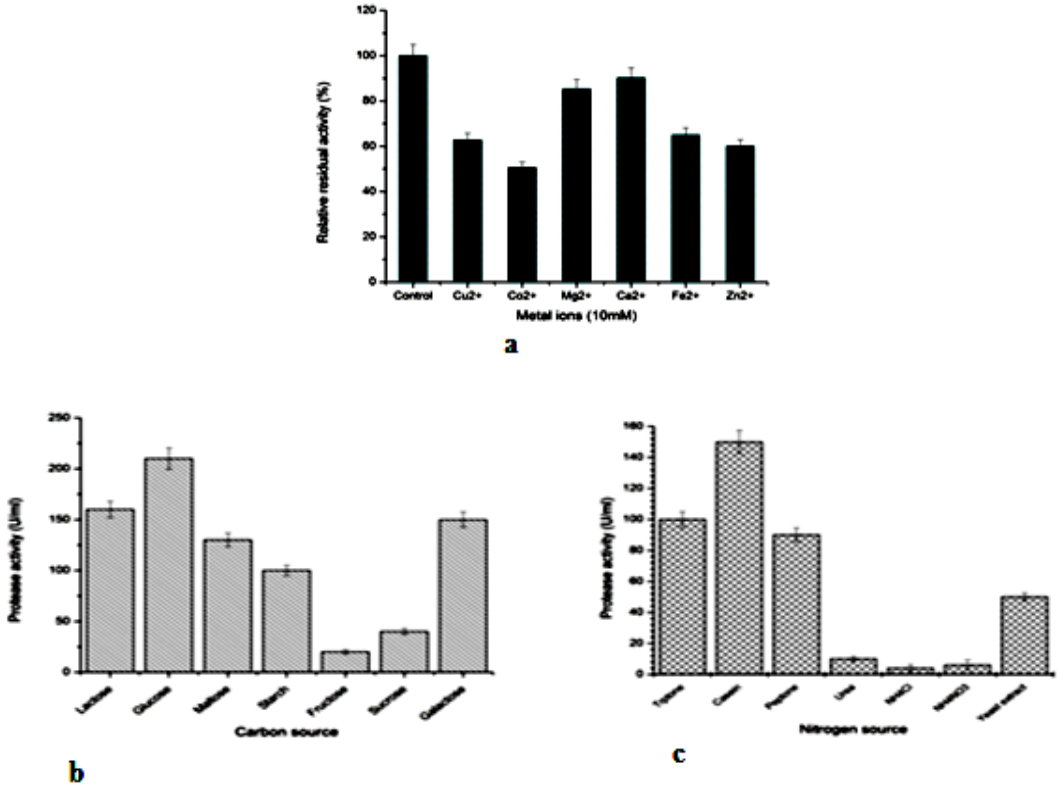

Fig.5. a. Effect of various metal ions on the purified protease activity of the enzyme b. Effect of various Carbon source, c. Nitrogen source on the purified protease activity of the enzyme

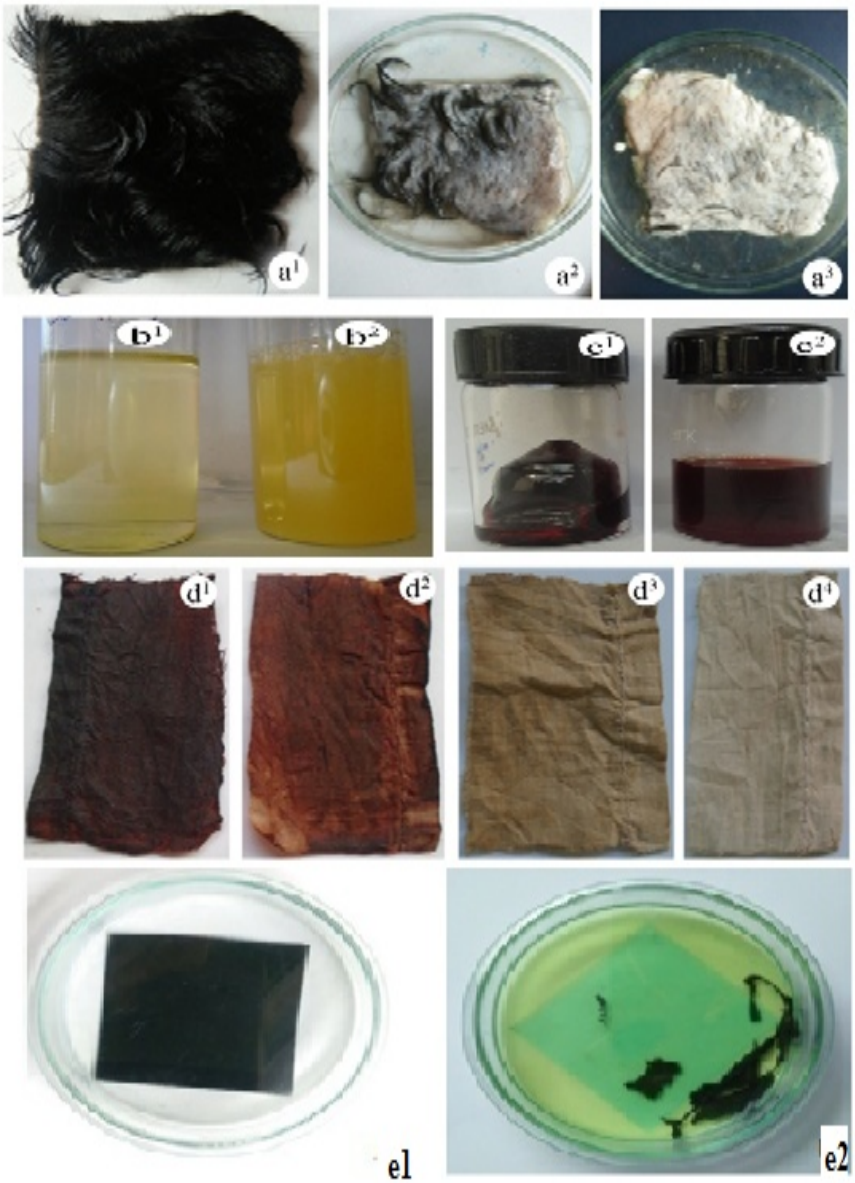

Fig.6. Power of protease from *M. luteus* on the digestion of natural proteins. Dehairing property of protease tested with sheep skin. a1 Control , a2 ,a3 sheep skin incubated after 6 and 10 hrs of incubation at room temperature; The protease was incubated with coagulated egg albumin at 37ºC for 6 hrs (b1 control, b2 after 6 hrs). Blood clot removal by protease; c1 control, c2 after 12 h of with protease at room temperature. Wash performance assay of alkaline protease; d1 control, goat blood stain removed from cotton cloth after 3, 6, 9 hr (d2, d3 ,d4 respectively) of incubation at room temperature without the aid of detergents. Decomposition of gelatinous layer from X-ray film e1. Control, e2Purified Protease Enzyme with film after 3 hrs.

Table. 1. Partial purification and yield of the protease from *Micrococcus luteus*

S.no	Steps	Total activity (U/ml)	Total protein (mg/m)	Specific activity (U/mg)	Purification fold	Yield%
1	Crude enzyme	2200	2.01	1095	1	100
2	Ammonium sulphate precipitation 80%	1470	1.41	1542	1.5	66.8
3	Dialysis	1071	0.43	2490	2.5	48.6

Table . 2 . Protease activity and stability of pH were performed as triplicates. Mean Standard Deviation was calculated

pH	Activity (U/ml)	Stability (%)
2	50 ± 2.5	8 ± 0.4
3	110 ± 5.5	11 ± 0.6
4	150 ± 7.5	15 ± 0.8
5	210 ± 10.5	20 ± 1.0
6	449 ± 22.5	31 ± 1.6
7	600 ± 30.0	59 ± 3.0
8	1005 ± 50.3	90 ± 4.5
9	700 ± 35.0	75 ± 3.8
10	600 ± 30.0	66 ± 3.3
11	503 ± 25.2	59 ± 3.0
12	300 ± 15.0	50 ± 2.5

Table . 3. Protease activity and stability of temperature were performed as triplicates. Mean Standard Deviation was calculated

Temperature (^{o}C)	Activity (U/ml)	Stability (%)
20	310 ± 15.5	98 ± 4.8
30	640 ± 32.0	85 ± 4.0
40	915 ± 45.8	65 ± 3.3
50	759 ± 38.0	60 ± 3.0
60	591 ± 29.6	42 ± 2.1
70	515 ± 25.8	31 ± 1.6
80	312 ± 15.6	25 ± 1.3

CONCLUSION

The work presented here described the screening and molecular characterization of a marine sediment bacterium capable of producing very active protease of industrial importance. The protease produced by *M. luteus* MG603678 has prospective industrial applications this strain can be utilized as bio-catalysts in complete removal of goat skin, anticoagulant of egg albumin and blood, blood stain removal within a short time sans damaging small particle structure and also a potential in recovering silver from X-ray films. Therefore, we concluded that this protease from marine *M. luteus* can be applied in diverse industries due to its multi-faceted potential and eco-friendly approach.

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