



## Bio-production of alkaline protease by *Trichoderma longibrachiatum* and *Penicillium rubidurum* using different agro-industrial products

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### Abstract

Alkaline protease being active in neutral to alkaline pH has huge demands in food, detergent, leather and pharmaceutical industries. Its production from agro-industrial wastes not only lowers the production costs but also reduces the environmental problems. Hence, the present study aimed to search for new potential microbes, which can produce alkaline protease enzyme, to meet the industrial demands. In this study, 13 fungal spp. were isolated on potato dextrose agar medium (PDA) from mangrove soil through serial dilution, and then were streaked on the skim milk agar medium for qualitative screening of protease production. Out of 13 fungal spp.; only 7 spp. were able to produce proteolytic zones through the proteolytic assay. The Relative enzymatic index (REI) value (Zone diameter/Colony diameter) of all the fungal isolates that produced proteolytic zones on skim milk agar medium was evaluated. Only 2 fungal isolates which showed maximum REI value were selected, and then identified morphologically and molecularly as *Trichoderma longibrachiatum* (Accession no. MF144551) and by *Penicillium rubidurum* (Accession no. MF144561). Submerged fermentation was carried out using different agro industrial substrates to quantify for protease production, where the supernatants obtained were used for alkaline protease estimation. Among the different tested substrates, soybean powder and wheat bran were the most suitable substrates for maximum protease production by *T. longibrachiatum* (233.78±7.12 U/ mg) and *P. rubidurum* (228.61±11.13 U/ mg), respectively. The partial purified enzyme from these fungi showed maximum proteolytic potentials at pH 8.0 (*P. rubidurum*) and pH 9.0 (*T. longibrachiatum*), with optima temperature of 40 °C. Among the tested heavy metals, only Mn<sup>2+</sup> expressed marginal enhancement of the protease enzyme activity.

**Keywords:** Alkaline protease, Agro-industrial waste, Fermentation, Fungi



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## 1. Introduction

Proteases are group of hydrolytic enzymes that are able to break down the peptide bonds in proteins ([Sharma \*et al.\*, 2019](#); [Karray \*et al.\*, 2021](#)). Alkaline proteases with higher stability in alkaline condition have more demand in the detergent industry ([Elgammal \*et al.\*, 2020](#)). To meet the industrial demands for alkaline proteases, search for new potential microbes is very important ([Karray \*et al.\*, 2021](#)). Proteases are produced by various microorganisms, however not all of these enzymes are able to meet the requirements of the industrial demands ([Sharma \*et al.\*, 2017](#)). Recently, [Beyan \*et al.\*, \(2021\)](#) reported that among the protease producing microorganisms; fungi are considered as the commercial protease producers. Although many fungal species that belong to several Genera including; *Aspergillus*, *Trichoderma*, *Rhizopus*, *Mucor* and *Penicillium* were reported for protease production; however, its production at a commercial level was not examined yet ([Benluvankar \*et al.\*, 2016](#)). Unlike other microorganisms, protease production by fungi depends upon several criteria including; the morphology, age of fungal culture, media composition, moisture content and heavy metal concentration, all affect fungal protease production, as revealed by [Nadeem \*et al.\*, \(2020\)](#). In addition, cost effective protease production by the fungal spp. is an important thing in the competitive enzyme markets, which has to be considered on searching for new potent fungal strains, as reported in the previous study of [Germano \*et al.\*, \(2003\)](#). Hence, the substrates to be manipulated for protease production must be of low costs, with high carbon and protein contents ([Fernandez, 2002](#)). Accordingly, the inexpensive and widely available agricultural solid wastes could be used as better substrates for microbial protease production ([Ortiz \*et al.\*, 2016](#)).

Keeping the above in view, the objective of the present study was to explore the potential fungal

species that can produce the alkaline protease enzyme from the low cost agro-industrial products.

## 2. Materials and methods

### 2.1. Isolation of fungi from the soil samples

Collection of soil samples were done from the different sites of Bhitarkanika mangrove forest, Odisha, India. The collected soil samples immediately after aseptic collection were brought to microbiology laboratory and kept at 4 °C ([Sethi \*et al.\*, 2013](#)). Each soil sample was serially diluted and then poured into potato dextrose agar (PDA) Petri plates, according to [Sudarkodi \*et al.\*, \(2015\)](#). The plates were incubated at 30 °C for 2-3 d, and then fungal colonies recovered were preserved in PDA slants at 4 °C for further studies.

### 2.2. Qualitative screening of fungal isolates for alkaline protease production potential

For qualitative screening of protease production, all the fungal isolates were streaked longitudinally on the surface of skim milk agar medium (g/l: Peptone, 5g; Beef extract, 3g; NaCl, 5g; skim milk 1%, Agar, 15g; pH 8.0) with slight modification to [Suryawanshi and Pandya, \(2017\)](#), and then incubated at 30± 1°C for 96 h. After incubation, fungal colonies giving hydrolyzing zones around the streaks were considered as protease producers. The width of the hydrolyzed zone around the streak versus the width of the colony was measured, and then the relative enzymatic index (Zone diameter/Colony diameter) was calculated ([Reshma and Pradeep, 2017](#)). Fungal isolates that showed widest zones of hydrolysis were selected for further studies.

### 2.3. Identification of the selected fungal isolates

Identification of the selected fungal strains that produced the widest proteolytic zones on skim milk agar medium was carried out based on their

morphological characteristics, and on lacto phenol cotton blue staining, following the standard methods of [Gilman, \(1998\)](#). For molecular identification, genomic DNA of the selected fungi was isolated following the standard method of ([Moller \*et al.\*, 1992](#)). The ITS rDNA gene was amplified through Polymerase chain reaction (PCR) (Biorad T100 Thermal Cycler) using both of; ITS1 (5'- TCCGTAGGTGAAC CTGCGG - 3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers ([White \*et al.\*, 1990](#)). Amplification was carried out using 12.5 µl of 2× PCR Master Mix containing dNTPs; PCR Buffer and Taq DNA Polymerase (Fermentas), 12.5 µl of a solution containing 200 nM of each primer, 100 ng of the template DNA and sterile DNase, RNase free water. The PCR reactions were carried out for an initial denaturation (94 °C for 45 s), annealing (55 °C for 45 s) and extension (72 °C for 60 s); each of 35 cycles with an initial hot start (94 °C for 15 min.) and a final extension (72 °C for 10 min.). The PCR products were analysed by agarose gel electrophoresis; documented in Gel doc system (Biorad), eluted and then purified by gel purification kit (Qiagen). After purification, the PCR product was sequenced in an automated ABI 3730×1 genetic analyzer system (Applied Biosystems, USA) using the BigDye Terminator v3.1 Cycle sequencing Kit following the manufactures protocol, and then visualized after ethidium bromide staining. The sequences were matched through BLAST search operation at NCBI GenBank, and then the obtained sequences were aligned with similar sequences of other fungi retrieved from databases using CLUSTAL W ([Thompson \*et al.\*, 1997](#)). The evolutionary history was inferred using the Neighbor-Joining method and then a phylogenetic tree was constructed; using the neighbour-joining method using MEGA 4.0 ([Saitou and Nei, 1987](#)).

## 2.4. Submerged fermentation production of alkaline protease enzyme

### 2.4.1. Preparation of fungal inocula

Fungal spore suspension was prepared from 7 - 10 d old culture of the selected fungal culture following

the standard method conducted by [Sethi \*et al.\*, \(2016a\)](#). About 15 ml of sterile saline water that was mixed with 0.1 % of tween 80 was added to the selected fungal culture; conidia were scraped from the surface of the culture and then diluted to obtain the desired conidial suspension concentration of  $2 \times 10^7$  conidia/ml.

### 2.4.2. Qualitative production of alkaline protease

According to the modified method of van den [Hajji \*et al.\*, \(2007\)](#), submerged fermentation for alkaline protease production was carried out using the fermentation medium containing (g/ l):  $\text{KH}_2\text{PO}_4$  1.0;  $\text{Na}_2\text{HPO}_4$  0.8;  $\text{CaCl}_2 \cdot 7\text{H}_2\text{O}$  0.4;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5; NaCl 0.3;  $\text{ZnCl}_2$  0.1 and different agro-industrial products of 10 g/ l (pH 8.0) each. The used agro-industrial products included; wheat bran (WB), black gram peels (BGP), green gram peels (GGP), horse gram peels (HGP) and gram peels (GP), which were collected from huller machine shop and different local markets of Bhubaneswar; washed, air dried and then dried in a hot air oven at 70 °C for 2 h ([Dutta \*et al.\*, 2019](#)). Peels were ground to powder forms to be used in the fermentation medium. Beside the above mentioned agro-industrial products; skimmed milk powder were also used for screening of protease production. An aliquot of 0.5 ml of the prepared conidial suspensions was inoculated individually to 50 ml of the pre-sterilized fermentation medium, and then shaker incubated at  $30 \pm 1^\circ\text{C}$  for 96 h at 150 rpm ([Sethi \*et al.\*, 2016b](#)). After incubation, centrifugation of the growing culture was carried out at 10,000 rpm in a cooling centrifuge at 4 °C. After filtration using Whatman filter paper (no. 1), the supernatants obtained were used for estimation of alkaline protease activity.

The total protein present in the crude enzyme sample was estimated according to the method adopted by Lowery *et al.*, (1951), using Bovine serum albumin as a standard. The amount of fungal biomass obtained was determined according to [Sethi \*et al.\*, \(2016b\)](#). All the assays and estimations were carried out in triplicates.

### 2.4.3. Quantitative assay for alkaline protease production

Quantitative estimation of amounts of protease produced by the selected fungal isolates was determined according to the standard method described by [van den Hombergh \*et al.\*, \(1997\)](#). Approximately 450 µl of enzyme sample diluted with 0.1M tris-HCl buffer (pH 8.0) was incubated with 50 µl 1% (w/v) of azocasein at 40°C for 30 min. After incubation, the reaction was stopped using 500 µl of 10% (w/v) Trichloroacetic acid (TCA), and then incubated at 0°C. After 30 min. of incubation, the reaction mixture was centrifuged at 6000 rpm for 5 min., and then the supernatant was used for enzyme estimation according to the method of [Lowry \*et al.\*, \(1951\)](#). A single unit (U) of protease activity was defined as the amount of enzyme capable of releasing 1 µg of tyrosine\ minute.

### 2.5. Partial purification of alkaline protease

Purification of the alkaline protease enzyme was performed using ammonium sulphate precipitation method ([Sethi \*et al.\*, 2016a](#)). Ammonium sulphate solution (70 % w/v) was added gently to the crude enzyme and then mixed using a magnetic stirrer at 4°C. The precipitated protein was recovered by cold centrifugation at 10,000 rpm for 10 min. at 4°C. Further, the precipitated protein was dialyzed overnight at 4°C against 0.01 M tris-HCl buffer (pH 8.0). After partial purification, the protease activity and total protein contents were measured using the standard procedure described above.

### 2.6. Characterization of the partially purified alkaline protease

Characterization of the partially purified protease with respect to pH, temperature and metal ions was carried out following the methods conducted by [Li \*et al.\*, \(2014\)](#) as follows:

#### 2.6.1. Effect of pH on the alkaline protease enzyme potential

Several buffers including; citrate-phosphate buffer (pH 4.0-6.0), phosphate buffer (7.0), Tris-HCl buffer (pH 8.0), glycine–NaOH (9.0-10.0) and carbonate bicarbonate buffer (pH 11.0), were used to study the effect of pH on the partially purified enzyme, in reference to [Benmard \*et al.\*, \(2016\)](#). Maximum enzyme activity determined within the pH range was expressed as 100 %, and then the residual potentials were calculated accordingly ([Benmard \*et al.\*, 2019](#)).

#### 2.6.2. Effect of temperature on the partially purified alkaline protease potency

To determine the effect of temperature on the partially purified protease activity, the enzyme was incubated for 30 min. at different temperatures (25-60 °C); using azocasein as a substrate. After incubation, the enzyme was cooled and the protease activity was determined following the standard method of [van den Hombergh \*et al.\*, \(1997\)](#). The enzyme activity recorded without heating was considered as 100 %, where the residual activities were then evaluated, in reference to [Benmard \*et al.\*, \(2019\)](#).

#### 2.6.3. Effect of heavy metals on the activity of the alkaline protease enzyme

The partially purified enzyme was incubated individually with the corresponding metal ions solutions including: CuCl<sub>2</sub>, FeCl<sub>3</sub>, Pb(NO<sub>3</sub>)<sub>2</sub>, MnCl<sub>2</sub>, ZnCl<sub>2</sub>, CoCl<sub>2</sub>, NiCl<sub>2</sub> CdCl<sub>2</sub> and K<sub>2</sub>CrO<sub>4</sub> at 0.5 mM concentration for 30 min. at 40°C before adding the azocasein substrate. The proteolytic activity was determined according to the standard conditions described above ([van den Hombergh \*et al.\*, 1997](#)). The control enzymatic activity (without the metal inhibitor) was considered as 100 %, whereas the residual enzyme potential was estimated [Benmard \*et al.\*, \(2019\)](#).

### 2.7. Statistical analysis

Statistical analysis was performed using SPSS version 10 for windows (SPSS Inc; Chicago, IL, USA). Test results were demonstrated throughout the mean and standard errors of the replicates of each variable, in reference to [Zar, \(1984\)](#).

### 3. Results

#### 3.1. Isolation of fungi

A total 13 fungal isolates were recovered from the soil samples. Colony morphology of the fungal isolates varied from elevated; smooth, rough, circular, opaque, punctiform, and were white; grayish green, dark and black colored colonies on PDA plates. The isolated fungal spp. were assigned different symbols from of NBS-1-NBS-6 and ISB-1- ISB-7.

#### 3.2. Qualitative screening for alkaline protease production by fungi

Fungi isolated from mangrove soil have been screened

on skim milk agar medium to check their protease production efficacy. Out of the 13 morphologically distinct fungal isolates; 7 of these isolates that produced halo zones on Skim milk agar medium were considered as protease producers. The clear zones and colony diameters of these isolates were measured (zone size by colony size diameter) (Table 1), and then the average relative enzymatic index (REI) values ( $p < 0.05$ ) were evaluated. The REI values ranged from 1.05 cm in isolate NBS-4, to 1.64 cm in isolate ISB-7, demonstrating that all the 7 isolates had the abilities to degrade protease. On the basis of maximum REI values; the two fungal isolates i.e., ISB-7 (1.64 cm) and NBS-5 (1.5 cm) were selected for further quantitative studies.

**Table 1:** Screening for protease activity of the fungal isolates on Skim milk agar medium

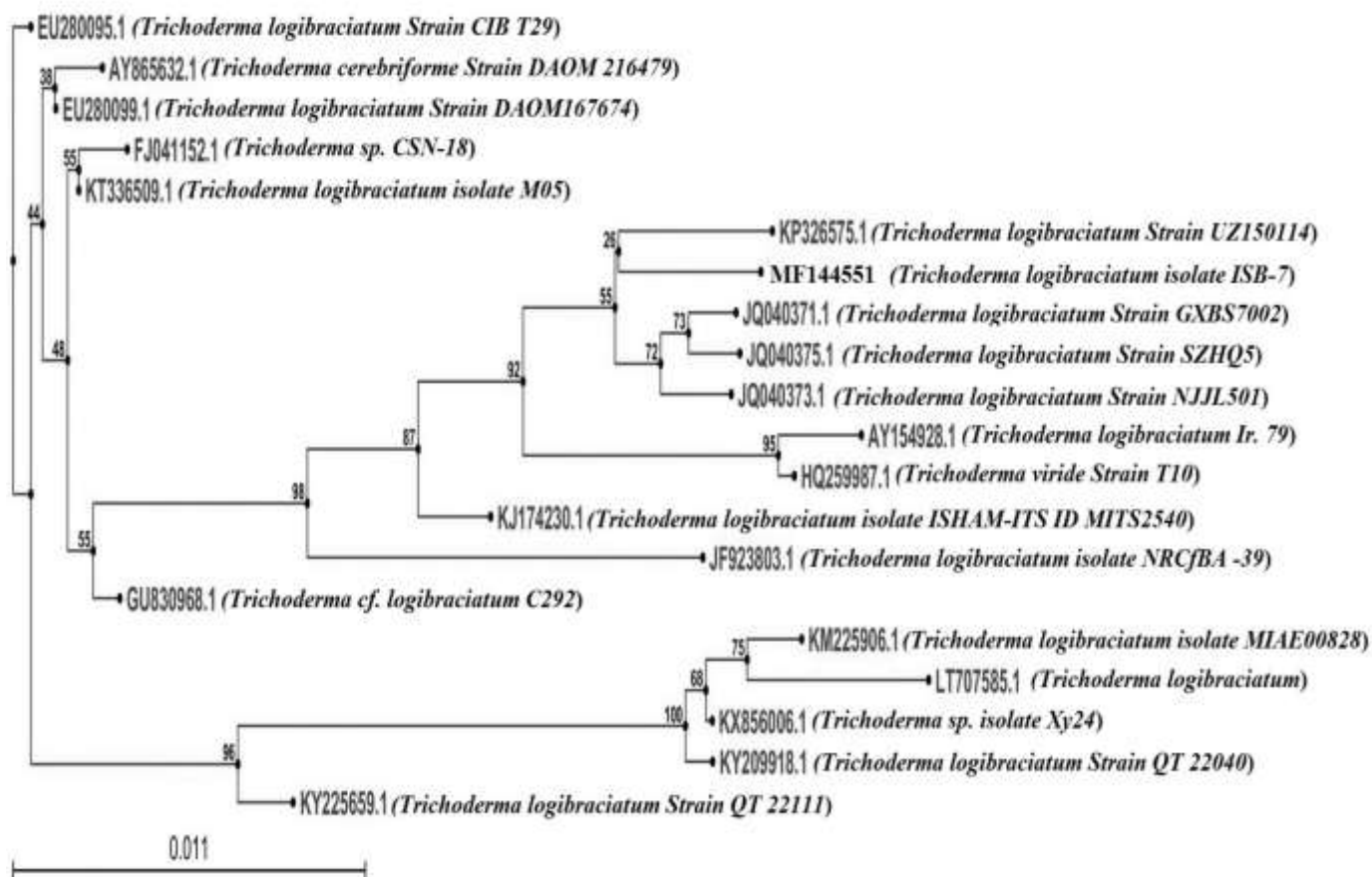
Name of the strain	Colony diameter (mm)	Zone diameter (mm)	REI value (Zone diameter/Colony diameter)
NBS-1	10± 0.33	ND	NA
NBS-2	7± 0.11	8± 0.43	1.14
NBS-3	9± 0.23	ND	NA
NBS-4	17± 0.13	18± 0.17	1.05
NBS-5	20± 0.39	30± 0.33	1.5
NBS-6	19± 0.57	ND	NA
ISB-1	17± 0.21	28± 0.81	1.64
ISB-2	16± 0.27	ND	NA
ISB-3	9± 0.11	10± 0.23	1.11
ISB-4	23± 0.21	ND	NA
ISB-5	21± 0.83	24± 0.13	1.14
ISB-6	24± 0.21	27± 0.31	1.12
ISB-7	17± 0.33	ND	NA

Where; \*Values are the mean colony and Zone diameters (mm) of three replicates; ±: SD of the recorded results ( $p < 0.05$ ). NBS-1 - NBS-6 and ISB-1 - ISB-7: represent the isolated fungal strains; REI= Relative enzymatic index; ND= Not detected; NA= Not applicable.

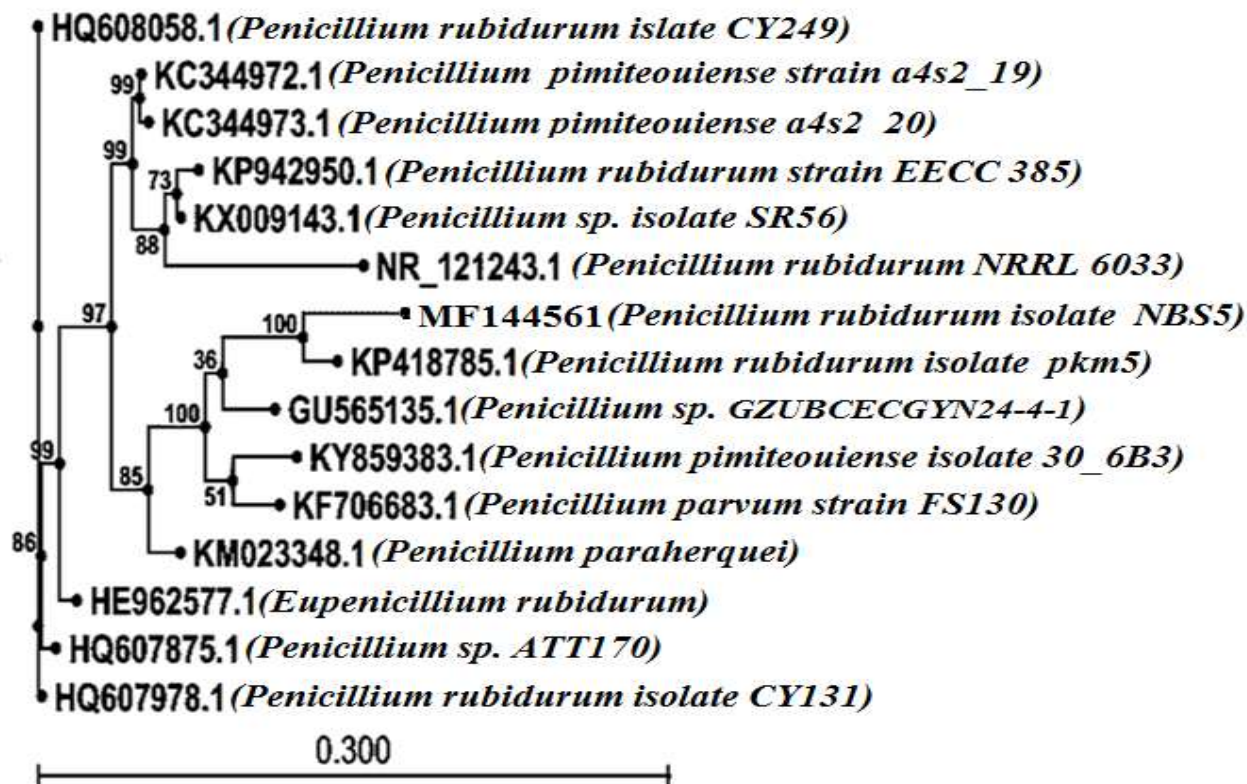
### 3.3. Identification of the selected protease producing fungi

The selected protease producing fungal isolate ISB-7 was off white at the beginning that changed to greyish green after 72 h of incubation on PDA agar plates. Similarly, the isolate of NBS-5 was white in color at the beginning that changed to light green with further incubation. The fungal conidiophores were observed using a light microscope after staining with lacto phenol cotton blue. Based on the morphological and microscopical characterization, the promising fungal isolate ISB-7 was tentatively identified as

*Trichoderma* sp., whereas the isolate of NBS-5 was identified as *Penicillium* sp. Furthermore, through ITS rDNA gene sequencing followed by BLAST and Phylogenetic tree analysis; the genus and species of the selected strains were confirmed. The isolate of ISB-7 showed 99 % similarity with the *Trichoderma longibrachiatum* (Fig. 1), while the isolate of NBS-5 presented 99 % similarity with *Penicillium rubidurum* (Fig. 2). The ITS rDNA gene sequences of both isolates were deposited in NCBI database with Gene bank accession numbers of MF144551 for *T. longibrachiatum*, and MF144561 for *P. rubidurum*, respectively.



**Fig. 1:** Un-rooted tree constructed by using the neighbor-joining method; showing the phylogenetic relationship of *Trichoderma longibrachiatum* isolate ISB-7 to the other *Trichoderma* spp.

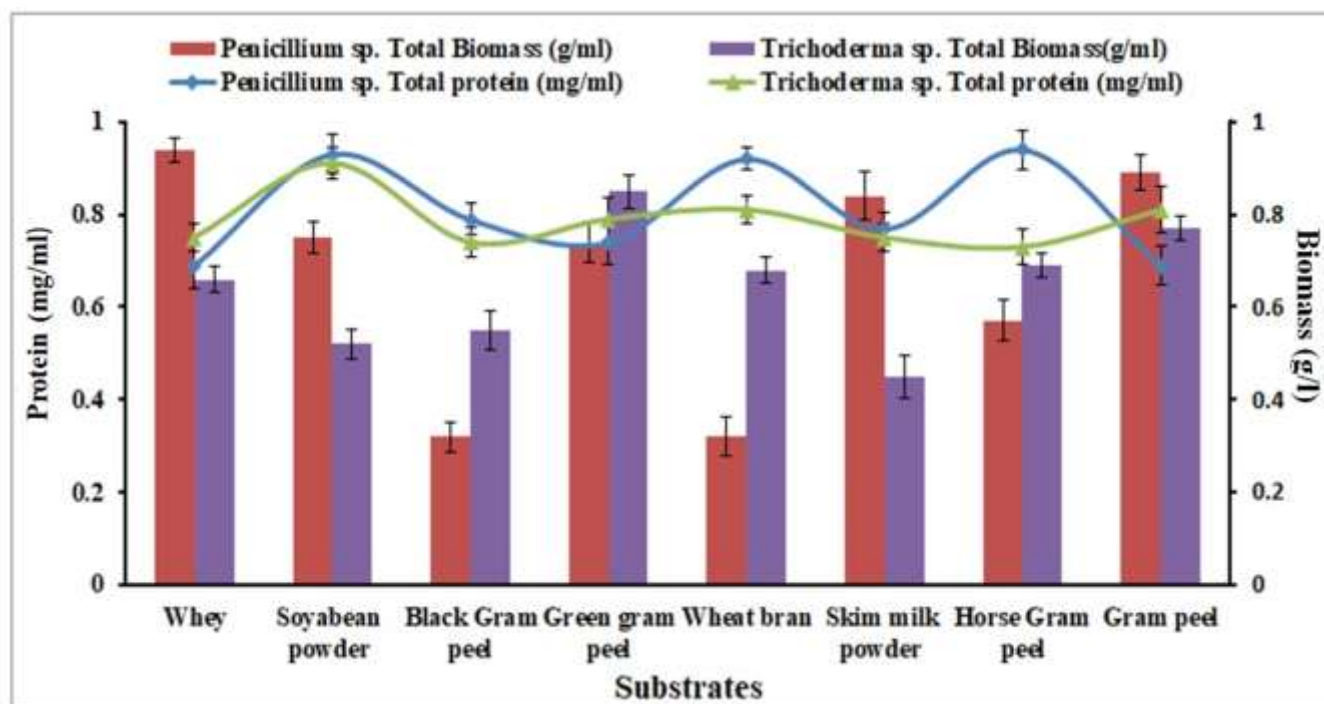


**Fig. 3:** Un-rooted tree constructed by using the neighbor-joining method; showing the phylogenetic relationship of isolate *Penicillium rubidurum* NBS-5 to the other *Penicillium* spp.

### 3.4. Quantitative production of alkaline protease using agro-industrial substrates

Several agro-industrial substrates were used to screen for the best substrate for maximum protease enzyme production. Among the various agro-industrial substrates studied, maximum protease production ( $233.78 \pm 7.12$  U/ mg) was recorded in the culture medium supplemented with soybean powder as a substrate; on inoculation with *T. longibrachiatum* ISB7 (Fig. 3). On the other hand, maximum protease production ( $228.61 \pm 11.13$  U/mg) obtained in the fermentation medium inoculated with *P. rubidurum* NBS5 was recorded on using wheat bran as a substrate. Compared to other agro-industrial substrates, minimum level of protease production ( $112.63 \pm 4.42$  U/mg) was recorded when gram peel was used as a substrate in the fermentation medium (Fig. 3). All the above

assays were run parallel in triplicates, and the obtained results differed significantly at  $p < 0.05$ . The recorded total protein of the crude extracts and biomass content were different when fermentation was carried out using different substrates. Maximum ( $p < 0.001$ ) protein content ( $0.94 \pm 0.043$  mg/ ml) was detected in the crude enzyme extract of *P. rubidurum*; when fermentation was carried out using horse gram peels as a substrate, whereas maximum biomass ( $0.94 \pm 0.027$  g/ ml) was obtained in the fermentation medium supplemented with whey (Fig. 4). Meanwhile, it was also observed that maximum biomass ( $p < 0.001$ ) of *T. longibrachiatum* was obtained on using green gram peel ( $0.85 \pm 0.036$  g/ ml) as a substrate, while maximum protein content ( $0.91 \pm 0.033$  mg/ ml) with recorded on using black gram peel, compared to the other substrates used during this assay ( $p < 0.001$ ).



**Fig. 4:** Total protein content and biomass produced by fungi in fermentation media; using different agro-industrial substrates ( $p < 0.001$ )

### 3.5. Partial purification of the alkaline protease

As maximum protease production was observed when fermentation was carried out using soybean powder and wheat bran, hence crude enzymes extracted from the fermentation medium of these two substrates were selected for partial protease purification study. Results demonstrated that the partially purified protease from *T. longibrachiatum* exhibited protein content of 0.53 mg/ml with a specific activity of 334.96 U/mg, which corresponds to 1.43-fold purification and 83.44% yield ( $p < 0.05$ ) (Table 2). Similarly, partially purified protease from *P. rubidurum*, exhibited a protein content of 0.73 mg/ml with a specific activity of 260.27 U/mg, which corresponds to 1.13-fold purification and 90.43% yield ( $p < 0.05$ ) (Table 2).

### 3.6. Characterization of the partially purified alkaline protease

The partially purified enzyme was characterized using different experimental conditions including; different pH values, temperatures and heavy metals.

#### 3.6.1. Effect of pH

The influence of pH on activity of the partially purified alkaline protease was investigated in the pH range of 4.0-11. Results showed that the alkaline protease was active within the pH range of 5.0-11. The optimum pH for maximum protease activity ( $p < 0.01$ ) produced by *P. rubidurum* was observed at pH 8.0, whereas the maximum protease activity ( $p < 0.01$ ) produced by *T. longibrachiatum* was recorded at pH 9.0 (Fig. 5a). The enzyme activity declined with further increase in pH value.

#### 3.6.2. Effect of temperature

Analysis of the protease enzyme activity at various temperatures showed that the enzyme from



both of *P. rubidurum* and *T. longibrachiatum* was a mesophilic protease, remaining active at temperatures range of 25- 45°C, while the optimal residual activity was detected at 40 °C ( $p < 0.05$ ). The enzyme activity declined at temperature above 45°C (Fig. 5b), indicating the conformational instability of the enzyme at high temperatures.

### 3.6.3. Effect of different metal ions

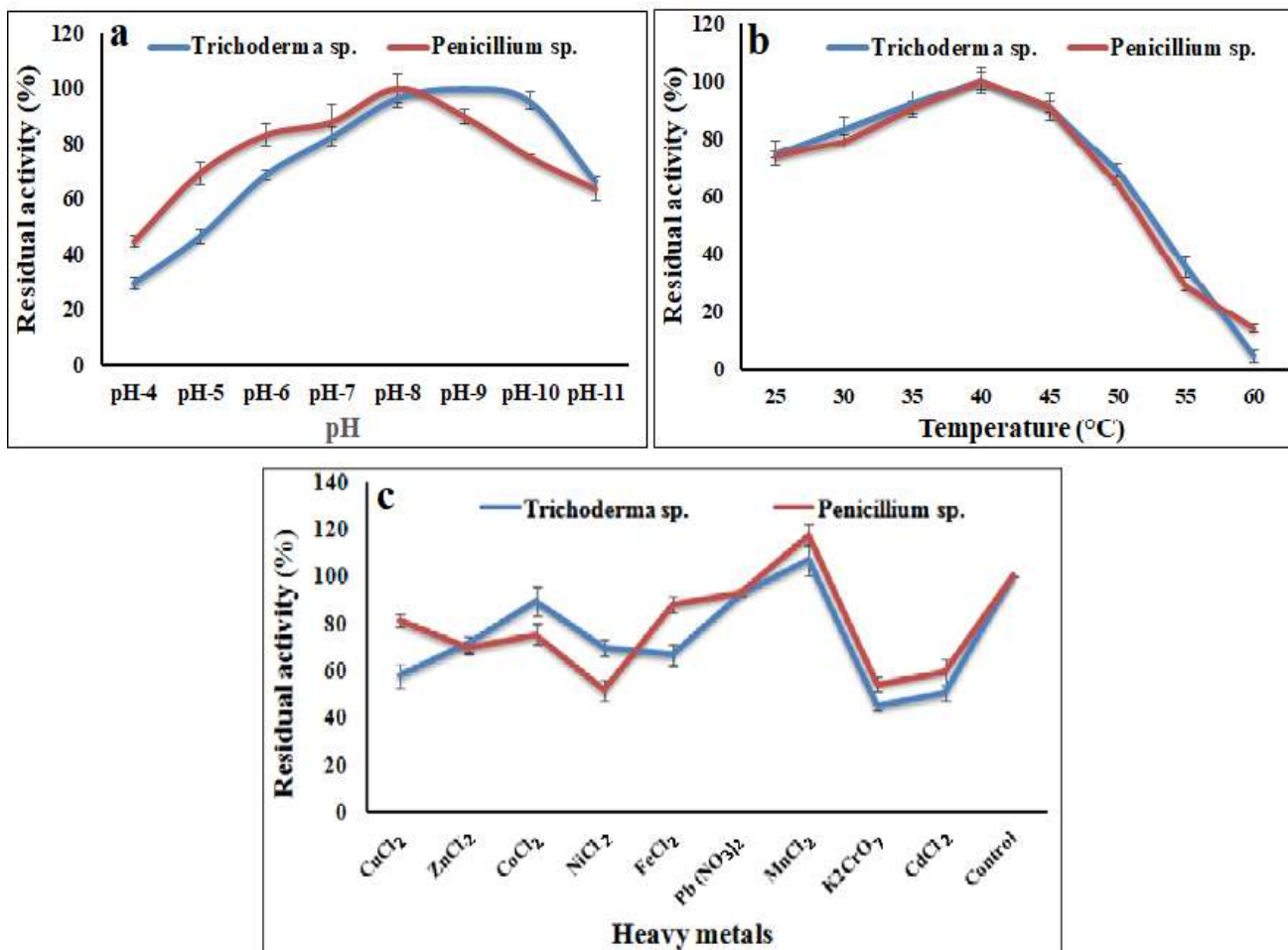
The effect of metal ions on enzyme activity is illustrated in Fig. (5c). In this study, metal salts such

as; Cd, Cu, Cr, Ni, Zn, Pb, Fe and Co revealed their inhibitory effects on protease production, compared to the controls. The  $Mn^{2+}$  heavy metal ion expressed marginal positive regulation on alkaline protease production. As shown in Fig. (5c), the catalytic activity of the partially purified alkaline protease was improved by using  $Mn^{2+}$ , with maximum increase in activity of 17 % and 6 % ( $p < 0.05$ ) recorded by *P. rubidurum* and *T. longibrachiatum*; respectively, compared to the controls.

**Table 2:** Partial purification of the alkaline protease produced by the selected fungal isolates in fermentation media supplemented with soya bean powder and wheat bran substrates

Fungus	Substrate	Protein extract	Protease activity (U/ ml)	Total protein (mg/ ml)	Specific activity (U/ mg)	Yield (%)	Purification (fold)
<i>T. longibrachiatum</i> (ISB-7)		Crude supernatant	212.74± 7.12	0.91	233.78	100	1
	Soybean powder	Ammonium sulphate precipitation (70%)	177.53± 0.39	0.53	334.96	83.44	1.43
<i>P. rubidurum</i> (NBS-5)		Crude supernatant	210.33± 0.35	0.92	228.61	100	1
	Wheat bran	Ammonium sulphate precipitation (70%)	190.21± 0.33	0.73	260.27	90.43	1.13

Where; \*Values are means of protease activity (U/ ml) of three replicates; ±: SD of recorded results ( $p < 0.05$ )



**Fig. 5:** Effect of (a): pH; (b): temperature and (c): heavy metals on the activity of the partially purified alkaline protease produced by *T. longibrachiatum* and *P. rubidurum* ( $p < 0.01$ ). The Error bars represent the standard deviation.

#### 4. Discussion

In this study, 13 fungal spp. were isolated from mangrove soil of Bhitarkanika. Reports of fungal diversity from mangrove soil were also reported earlier by [Gilna and Khaleel, \(2011\)](#). Skim milk agar is commonly used to demonstrate proteolysis by microorganisms capable of hydrolyzing casein. A proteolytic microorganism uses the enzyme caseinase to hydrolyse casein and form soluble nitrogenous compounds that are displayed as clear zones around the colonies. Out of the 13 morphologically distinct fungal spp.; 7 fungal isolates produced halo zones on skim milk agar medium, thus were considered as protease

producers. Currently, the recorded average REI value ranged from 1.05-1.64, similar to the REI obtained by [Reshma and Pradeep, \(2017\)](#), who observed maximum REI value of 1-2. On the basis of maximum REI values and protease production abilities in the fermentation media, two fungal isolates mainly; ISB-7 and NBS-5 were selected for further studies and then identified. Identification was carried out following the standard methods of "A Manual of Soil Fungi" ([Gilman, 1998](#)), and then confirmed through ITS rDNA gene sequencing followed by BLAST and Phylogenetic tree analysis; where the Genus and species of these strains were confirmed. Based on their micro-morphological characteristics and 18S rRNA gene sequences, the 2

selected isolates of ISB-7 and NBS-5 were identified as; *T. longibrachiatum* (Accession no. MF144551) and *P. rubidurum* (Accession no. MF14456), respectively. Similarly, proteases production abilities by *Penicillium* spp. and *Trichoderma* spp. have also been reported by several workers from different parts of the world ([De Marco and Felix, 2002](#); [Agrawal \*et al.\*, 2004](#); [Xie \*et al.\*, 2016](#)).

According to [Pandey \*et al.\*, \(2000\)](#), the selection of suitable agro-industrial substrate for enzyme production in fermentation process directly relies on cost and availability of this substrate. [Kumar and Takagi, \(1999\)](#) highlighted that each microorganism has its own conditions for maximizing the enzyme production. Current results of submerged fermentation indicated that protease production pattern varied with the type of the agro-waste used. Among the different agro-industrial substrates used; soybean powder was the best substrate for protease production by *T. longibrachiatum*, whereas wheat bran was the best protein substrate for *P. rubidurum*. [Mukhtar and Haq, \(2013\)](#) attributed the effectiveness of soybean as it contains significant amounts of proteins and other nutrients that are supportive for profuse growth and secretion of the proteolytic enzyme. Several previous studies have also used soybean as a substrate in the fermentation medium used for production of proteases ([Mariana \*et al.\*, 2008](#); [Radha \*et al.\*, 2008](#)). With respect to *P. rubidurum*, the reason for the highest enzyme yield recorded on using wheat bran might be attributed to the fact that wheat bran represents an adequate source of proteins, carbohydrates and minerals; needed for proper growth of the microorganism and biosynthesis of protease, as reported by [Mukhtar and Haq, \(2013\)](#). Moreover, wheat bran has a large surface area per unit volume suitable for good microbial growth on the solid/gas interfaces. Several other previous studies have also reported wheat bran as the best substrate for higher enzyme production ([Chutmanop \*et al.\*, 2008](#); [Shivakumar, 2012](#)). In the present study, maximum protease production recorded by *T. longibrachiatum* and *P. rubidurum*

was  $233.78 \pm 7.12$  U/ mg and  $228.61 \pm 11.13$  U/ mg, respectively. The present results of protease production are lower than those reported earlier by [Hajji \*et al.\*, \(2007\)](#). However, similar protease production level by other fungal isolates was also reported by the previous study conducted by [Li \*et al.\*, \(2014\)](#).

The ammonium sulphate-precipitated protease from *T. longibrachiatum* displayed specific activity of 334.96 U/ mg; representing 83.44 % yield and 1.43-fold purification. Similarly, the partially purified alkaline protease from *P. rubidurum* showed specific activity of 260.27 U/ mg; representing 90.43 % yield and 1.13-fold purification. Similar ranges of protease activities after partial purification from several fungal spp. were also recorded by [Sethi \*et al.\*, \(2016b\)](#).

Further, the partially purified enzyme was characterized using different experimental conditions such as; pH values, temperatures and heavy metals. On investigating the influence of pH on the activity of alkaline protease from *P. rubidurum*, the enzyme was active over a wide pH of 4.0–9.0 with an optimum activity at 8.0, whereas pH 9.0 was optimum for *T. longibrachiatum*. The protease activity sharply declined at pH above 10, and the enzyme lost nearly 35% of its activity at pH 11. The change of enzyme activity at different pH might result from a slight change in the enzyme conformation ([Hammes, 2002](#)). The present findings are in accordance with several earlier reports, who reported pH optima of 8.0–9.5 for proteases produced by *A. fumigates* ([Reichard \*et al.\*, 1990](#)), *A. parasiticus* ([Tunga \*et al.\*, 2003](#)) and *A. clavatus* CCT2759 ([Tremacoldi \*et al.\*, 2007](#)). Similarly, the protease activity by *Penicillium* sp. was obtained at pH 8.0 and temperature of 40°C, recorded during the previous work of [Xie \*et al.\*, \(2016\)](#). Conversely, the current protease activity recorded by *T. longibrachiatum* is higher than that reported earlier by [De Marco and Felix, \(2002\)](#) on *T. harzianum*,

who reported maximum alkaline protease activity at pH 8.0 and temperature of 37°C.

The optimum temperature for protease activity observed by both fungal strains in the present study was 40°C, which is lower than that reported by [Hajji \*et al.\*, \(2007\)](#), who observed optimum alkaline protease activity by *A. clavatus* ES1 at 50°C. The present findings are similar to those of *A. clavatus* CCT2759 ([De Marco and Felix, 2002](#)), *A. fumigatus* TKU003 ([Wang \*et al.\*, 2005](#)) and *A. fumigatus* CBS113.26 ([Larcher \*et al.\*, 1992](#)), with optima enzyme activities at temperatures between 37-42 °C.

The effect of specific metal ions on enzyme synthesis is solely relying on the microbial strain ([Xie \*et al.\*, 2016](#)). In the present study, pre-incubation of the fungal culture filtrates with various metal salts revealed the inhibitory effects of; Cd, Cu, Cr, Ni, Zn, Pb, Fe and Co metal ions, compared to the control protease enzyme of *P. rubidurum* and *T. longibrachiatum*. However, the heavy metal ion Mn<sup>2+</sup> exhibited positive regulatory effect on alkaline protease production. A previous study of [Agrawal \*et al.\*, \(2004\)](#) reported Mn<sup>2+</sup> as a known inducer and stabilizer of many enzymes through protecting them from conformational changes. Conversely, an earlier report highlighted that Mn<sup>2+</sup> decreased the activity of alkaline proteases from *A. clavatus* ES1 ([Hajji \*et al.\*, 2007](#)). In contradiction to the present report, [Ire \*et al.\*, \(2011\)](#) reported the positive effects of Ba; Ca, Co, Fe, Mn, Ni and Zn on protease production by *A. carbonarius*, recording highest protease secretion in the medium containing Fe.

## Conclusion

In the present report, alkaline protease produced from agro-industrial substrates showed pH optima of 9.0 by *T. longibrachiatum* and pH of 8.0 by *P. rubidurum*; with an optimum temperature of 40 °C. Moreover, the agro-industrial substrates such as soybean and wheat bran that recorded maximum protease enzyme activity when used as alternative

substrates; can be used for wide scale industrial production of this enzyme with reduced costs.

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## Conflict of interest

The authors have no conflict of interests that are relevant to the contents of this article.

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## Ethical approval

Non-applicable.

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