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Thermostable acidic protease production in *Aspergillus terreus* NCFT 4269.10 using chickling vetch peels

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Abstract

A newly isolated fungus, *Aspergillus terreus* NCFT4269.10, was employed in both solid state (SSF) and liquid static surface culture (LSSC) for the production of protease using different agro-residues. Among different substrates appraised, chickling vetch peels (CVP) supported the enhanced production of protease both at LSSC and SSF (499.99 ± 11 U/ml; 5266.8 ± 202.5 U/gds, respectively). In the presence of peptone (1%, w/v), leucine (5 mM/100 ml), Fe^{2+} (1 mM) and riboflavin (10 mg/100 ml) with a medium pH of 5.0 incubated at 30 °C for 96 h, 3-fold higher protease production was achieved in LSSC compared with control. Fermentation kinetics studies revealed that the highest specific growth rate of *A. terreus* was observed in fermentation medium supplemented with riboflavin (10 mg/100 ml), i.e., $256.45 \, \text{mg} \, l^{-1} \, h^{-1}$. The growth-associated coefficient of enzyme production (α) by *A. terreus* was maximal when protease was produced using Fe^{2+}. Further, the protease was purified to electrophoretic homogeneity and its molecular mass was determined as 23.8 kDa. The present strain suggests the potential utilization of inexpensive agro-residues (CVP) as medium components for the efficient industrial production using LSSC.
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Keywords: *Aspergillus terreus*; Chickling vetch peels; Liquid static surface fermentation; Protease; Solid-state fermentation

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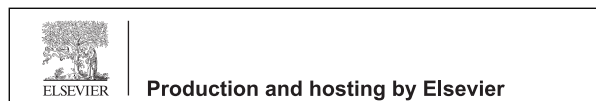
1. Introduction

As high-flying industrial enzymes, proteases catalyze the hydrolysis of peptide bonds present in proteins and can be categorized into two extensive groups, exopeptidases and endopeptidases, owing to the site of action. Depending on the presence of amino acids at the active site, these proteases can be further classified as serine, threonine, cysteine and aspartate proteases; metalloproteases; and glutamic acid proteases. Nonetheless, proteases can also be grouped into three types depending on their pH optima, i.e., acid proteases: pH optimum

below 7.0 (2.0–5.0), alkaline proteases: pH optimum above 7.0 and neutral proteases: pH optimum 7.0. The multifarious proteases of different types exhibit specificity in substrate, active site, catalytic mechanism, pH, temperature optima and stability profiles. Studies concerning the properties of these proteases are vital for the successful exploitation of biocatalysts in industries [1], such as detergents, leather processing, silver recovery [2], pharmaceuticals, food and feed processing, chemical industries, waste treatment [3] and amino acid mixtures resolution, with features for biotechnological applications [4]. Commercially, proteases are the most significant industrial enzymes, accounting for approximately 60% of the total global trade of enzymes [5].

Proteases of protozoa, bacteria, yeast and fungal origins degrade complex proteins for nutrition [6]. Nonetheless, proteases from fungal genera, including *Aspergillus*, *Penicillium, Cephalosporium*, *Mucor*, *Candida, Neurospora* and *Rhizopus*, have industrial significance. Amongst the many filamentous fungi, proteases from *Aspergillus* spp. have extensively been exploited in diverse industries, reflecting the activity of these enzymes at various pH levels and temperatures [7]. Acid proteases are produced from *Mucor miehei*, *Mucor hiemalis*, *Mucor racemosus* and *Mucor bacilliformis* [8]. Similarly, pepsin-like acid proteases are produced from *Aspergillus* [9] and *Rhizopus* species [10].

Fermentation expenses for the production of "bulkware" proteases under most conditions supports solid-state fermentation (SSF) compared with submerged fermentation (SmF), as SSF employs intricate, heterogeneous agricultural residues as substrates that are plentiful, economical and functional with inexpensive technology concerning sterility and regulation demands. Surprisingly, the process and downstream factors (constancy of the secreted enzymes at high temperature or extreme pH) have also been documented as superior in SSF [11]. Catabolite repression or protein degradation through proteases, an unavoidable dilemma in SmF is often reduced or absent in SSF. Furthermore, filamentous fungi are the finest tailored microorganisms for solid-state fermentation (SSF), reflecting the hyphal mode of fungal growth, tolerance to low water activity, high osmotic pressure conditions and competitiveness of these organisms in natural microflora for the bioconversion of solid substrates.

Thus, the aim of the present study was to determine the production and optimization of the fermentation and its purification conditions for proteases for significant employment in various industrial processes.

2. Materials and methods

2.1. Substrates and chemicals

In the present study, different agro-substrates were procured from the locality of Bhubaneswar, Odisha and transported in new steel containers to the laboratory. These substrates include mustard oil cake (MoC), neem oil cake (NoC), groundnut oil cake (GnoC), black gram peels (BGP), green gram peels (GGP), chickling vetch peels/grass pea peels (CVP), wheat bran (WB), pearl millet residues (PMR), finger millet waste (FMW), broken rice (BR), banana peels (BP), apple pomace (AP) and orange peels (OP). These materials were dried at $60\,°C$ for up to 48 h inside a hot air oven and powdered using a blender. Analytical reagent grade chemicals were purchased from Sigma, Hi-Media Limited, SRL Pvt. Limited and Merck India Limited.

2.2. Fungal spore inoculum preparation

The young slant culture of *Aspergillus terreus* NCFT 4269.10 (GenBank accession Number: KT222271.1), a protease-producing strain [12], was used for subsequent experiments. The pre-fermentation culture of *A. terreus* NCFT 4269.10 was developed with a final spore concentration of $5.0 × 10^8$ spores ml^{-1} at $30 ± 1\,°C$. Further, dilutions were obtained using double-sterilized water to generate a working spore concentration of $1 × 10^7$ spores ml^{-1}, as determined using a UV-VIS spectrophotometer [13].

2.3. Screening for selection of suitable substrate

The screening of pre-processed substrates (10 g) was performed using liquid static surface culture (LSSC) and solid-state fermentation (SSF). These substrates were used as the principal media components and 50 ml of sterilized medium formulated with either MoC, NoC, GnoC, BGP, GGP, CVP, WB, PMR, FMW, BR, BP, AP or OP as substrates inoculated with $1 × 10^7$ spores ml^{-1} from a seven-day-old pre-fermentation culture broth and incubated at $30 ± 1\,°C$ under static conditions. Similarly, solid-state fermentation was performed using the above substrates (5 g, w/w) under the conditions described above. After fermentation, both fermented media (LSSC and SSF) were processed for the recovery of crude protease and subsequently stored at $-20\,°C$ until further analysis. The biomass formed during LSSC was oven dried at $80\,°C$ for up to 24 h to determine the dry weight.

2.4. Optimization of the fermentation parameters for enhanced protease production

Environmental, nutritional and other fermentation parameters were established for the enhanced production of protease using CVP as the fermentation medium through SSF and LSSC. The optimized process parameters were pH (3–10), temperature (24–45 °C at 3 °C interval), incubation time (24–168 h), additional carbon sources (1.0%, w/w), nitrogen sources (organic and inorganic, 1.0%, w/w), amino acids (1.0 mM), metal ions (1.0 mM), antioxidant vitamins (10–50 mg/100 ml), growth hormones (0.0025 mg/g, w/w), combined agrowastes, inoculum size (2–10%) and initial moisture content (20–100%). The effect of soaking time on the fermented products (1–96 h), repeated extraction and various extractants were also standardized for the easy, proficient and economic down streaming of the protease. The adopted approach was to scale up one variable-at-a-time (OVAT), independent of the other factors and consequently, the established conditions were adopted in subsequent experiments.

2.5. Purification of the protease

The crude protease (∼500 ml) was precipitated through the gradual addition of 40–80% ammonium sulphate $[(NH_4)_2SO_4]$ with constant stirring using a magnetic stirrer at 4 °C for up to 24 h. Each precipitate was separated from the supernatant through centrifugation at $10,000 \times g$ for 15 min at 4 °C. After centrifugation, the supernatant was decanted, and the solid precipitate was dissolved in phosphate buffer (pH 6.5) at a ratio of 0.1 g/ml to obtain 10-times more concentrated enzyme solution [14]. Thereafter, the ammonium sulphate-precipitated enzyme solution was dialyzed for 24 h at 4 °C with constant stirring against a large volume (1 L capacity) of phosphate buffer (pH 6.5) for the complete removal of lower molecular weight metabolites and ammonium sulphate from the dialysate. To improve the solute exchange, the dialysis buffer was replaced every 2 h to obtain a new concentration gradient. Subsequently, the dialyzed enzyme was loaded onto a Sephadex G-100 column (2.5 cm × 70 cm) and eluted with 50 mM phosphate buffer (pH 6.5) at a flow rate of 1 ml min^{-1}. Fractions of 2 ml each were subsequently collected to estimate the protein concentration [15]. The fractions showing maximum absorption at 750 nm were collected and evaluated for enzyme activity. The enzyme-positive fractions with higher enzyme activity were pooled, lyophilized and stored at −20 °C until further characterization.

2.6. Analytical methods

2.6.1. Total protein determination

The total protein content of the crude and purified protease was estimated [15], using bovine serum albumin as the standard and expressed as μg of protein per ml of extract obtained after fermentation and purification.

2.6.2. Protease assay

Extracellular protease activity was determined according to the method of van den Hombergh et al. [16]. Each 450-μl sample was incubated with 50 μl of 1% (w/v) BSA (Sigma) in 0.1 M sodium acetate buffer (pH 4.0) at 37 °C. After 30 min of incubation, the reaction was terminated with 500 μl of 10% (w/v) trichloroacetic acid (TCA). After incubation at 0 °C for 30 min, the precipitated proteins were removed through centrifugation at 6000 rpm for 5 min and the absorbance of the TCA-soluble fraction was estimated as per Lowry et al. [15]. One unit (U) of protease activity was defined as the amount of enzyme that releases 1 μg of tyrosine in 1 min under the assay conditions.

2.6.3. SDS-PAGE and zymographic analysis of protease

The samples (crude and purified protease) were electrophoresed using 10% SDS-PAGE for molecular weight determination [17]. For visualization of the bands, the gels were stained in a staining solution [0.1% (w/v) Coomassie-Brilliant Blue R-250, 50% (v/v) methanol, 7% (v/v) glacial acetic acid and 43 ml MilliQ-distilled water] for 90 min at room temperature, followed by destaining with 30% (v/v) methanol, 7% (v/v) acetic acid and 63 ml MilliQ-distilled water until the background was clear. The relative positions of the bands were analyzed using a Bio-Rad Gel documentation system. Different molecular weight protein markers ranging from 7 to 175 kDa (Bangalore Genei Ltd.) were used for SDS-PAGE.

For the in-gel activity assay, a 10% non-denaturing gel containing 0.1% casein (casein was incorporated into the resolving gel prior to the addition of APS and polymerization) was run for 8–12 h in running buffer (50 mM Tris-0.1 M glycine, pH 8.3) at 4 °C with a 20 mA current. After running, the gel was rinsed in 0.25% Triton-X for 1 h and incubated 2 h to facilitate proteolysis in 0.1 M Tris-HCl buffer (pH 9) at 50 °C. The gel was stained for 1 h in a solution of Brilliant Blue R-250 and subsequently destained with methanol–acetic acid–water (4:1:5) until the background became clear [18]. The gel image was captured in a Bio-Rad Gel doc system.

Table 1
Protease production from *Aspergillus terreus* NCFT4269.10 in liquid static surface culture and solid-state culture systems using various agricultural wastes as substrates.

Fermentation medium	Cultivation regimen	Protease activity	Total protein	Biomass (g/50 ml)
MoC	LSSC	137.69 ± 1.28	2917.2 ± 2.90	0.203 ± 0.013
	SSF	4366.7 ± 37.45	21.755 ± 4.83	–
NoC	LSSC	431.05 ± 1.28	1723.32 ± 11.05	0.094 ± 0.007
	SSF	3171.3 ± 12.63	23.1 ± 5.59	–
GnoC	LSSC	395.9 ± 1.39	2105.54 ± 3.09	0.243 ± 0.002
	SSF	4253.8 ± 24.44	16.755 ± 3.61	–
BGP	LSSC	136.56 ± 1.76	540.55 ± 2.36	0.085 ± 0.002
	SSF	3140.63 ± 10.64	11.32 ± 2.29	–
GGP	LSSC	298.53 ± 1.15	1951.66 ± 2.87	0.157 ± 0.004
	SSF	6423 ± 13.35	9.33 ± 2.1	–
CVP	LSSC	499.99 ± 11	2308.32 ± 4.38	0.2108 ± 0.006
	SSF	5266.8 ± 202.5	16.534 ± 4.26	–
WB	LSSC	392.863 ± 1.12	1393.88 ± 1.58	0.080 ± 0.001
	SSF	2215 ± 17.57	3.97 ± 1.19	–
PM	LSSC	265 .03 ± 1.68	1579.99 ± 2.22	0.1412 ± 0.212
	SSF	2661.6 ± 85.42	9.233 ± 2.1	–
FM	LSSC	265.03 ± 1.68	1702.21 ± 4.14	0.032 ± 0.004
	SSF	2640.6 ± 85.4	9.7 ± 2.1	–
BR	LSSC	399.68 ± 1.67	870.54 ± 2.46	0.1981 ± 0.0812
	SSF	1323.2 ± 17.31	6.53 ± 1.58	–
BP	LSSC	0 ± 0.00	1234.99 ± 49.51	0.017 ± 0.044
	SSF	0 ± 0.00	2.32 ± 0.59	–
AP	LSSC	133.33 ± 1.45	859.98 ± 1.21	0.2117 ± 0.0217
	SSF	0 ± 0.00	1.96 ± 0.91	–
OP	LSSC	0 ± 0.00	861.66 ± 1.21	0.031 ± 0.065
	SSF	896.6 ± 9.66	9.33 ± 2.09	–
CONTROL*	LSSC	235.26 ± 1.21	1921.65 ± 5.97	0.131 ± 0.043

[a] The liquid static surface culture (LSSC) and solid state (SSF) experiments were performed for 96 h at 30 °C. The data represent the means ± SD of replicates ($n = 3$). {U/ml and μg/ml are used for LSSC; U/gds and mg/gds are used for SSF}[Weight of biomass (W3) = weight of biomass and filter paper (W1) − only weight of filter paper (W2)].
[b] Control refers to basal medium containing 1% (w/v) casein as a protease inducer.

2.7. Fermentation kinetics study of protease

For fermentation using agro-wastes from *A. terreus*, the logistic Eq. (A.1) and the Luedeking–Piret model (Eq. (B.2)) were employed for microbial growth and enzyme production, respectively.

$$\frac{dx}{dt} = \mu_{\max} \left\{ 1 - \left(\frac{x}{x_{\max}} \right) \right\} x \qquad (A.1)$$

$$\frac{dP}{dt} = \alpha \mu x - \beta x \qquad (B.2)$$

where dx/dt: biomass accumulation in the culture medium ($g\,l^{-1}\,h^{-1}$); dP/dt: enzyme accumulation in the culture medium ($U\,ml^{-1}\,h^{-1}$); x: biomass ($mg\,ml^{-1}$) at time (t); μ: specific growth rate (h^{-1}); $\mu_{\max}$: highest specific growth rate observed during batch culture ($mg\,l^{-1}\,h^{-1}$); x_{max}: maximum attainable biomass ($mg\,ml^{-1}$); α: growth-associated coefficient of enzyme production ($U\,g^{-1}$); β: growth-independent coefficient of enzyme production ($U\,g^{-1}\,h^{-1}$).

2.8. Statistical data analysis

All experiments were performed in triplicate ($n = 3$) and repeated three times. The samples collected from each replicate were analyzed for enzyme production, including enzyme activity and the optimization (scaled up) of the culture conditions. Each value represents an average of three parallel replicates. The ± and error bars indicate standard deviation among the replicates. For each individual experiment, one-way ANOVA was calculated using SPSS 16.0 workbook software. Least significant differences were also calculated using Duncan's new multiple range tests.

3. Results and discussion

3.1. Screening and selection of a suitable substrate

For the economical production and recovery of enzymes, many fermentation trends has been altered and process parameters have been optimized. Furthermore,
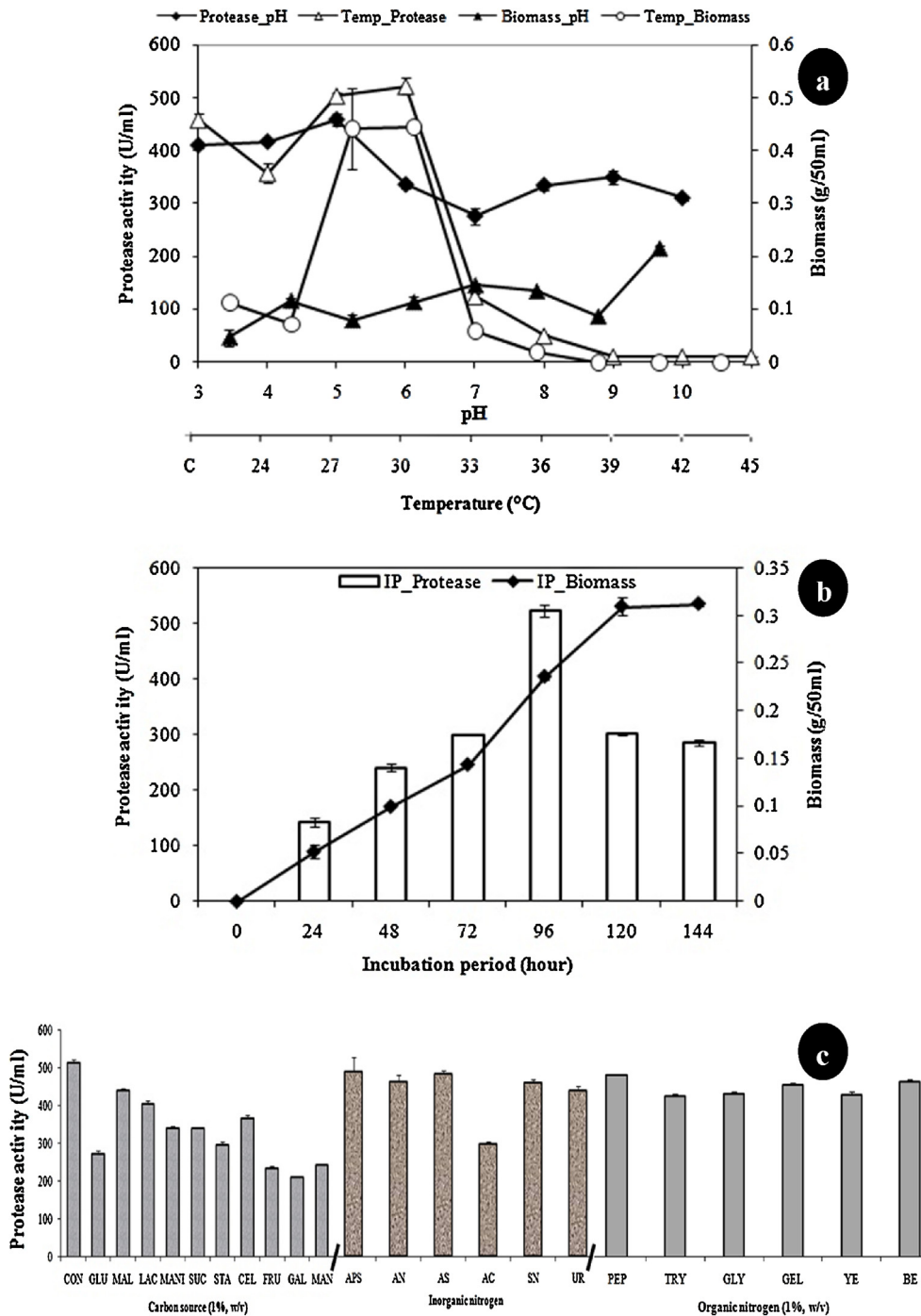
Fig. 1. (a) Effect of pH (3.0–10.0) and temperature (24–45 °C at 3 °C interval) on protease production and mycelia growth in liquid static surface culture (LSSC) in *A. terreus* NCFT 4269.10 (for pH, biomass of *A. terreus*: ▲; protease: ♦; for temperature, biomass of *A. terreus*: ◯; protease: △). (b) Effect of incubation time (24–144 h) on protease production and mycelia growth in liquid static surface culture (LSSC) in *A. terreus* NCFT 4269.10. (c) Effect of various carbon and nitrogen sources on the mycelia growth and protease production in *A. terreus*, where, CON: Control; GLU: Glucose; MAL: Maltose; LAC: Lactose; MANI: Mannitol; SUC: Sucrose; STA: Starch; CEL: Cellulose; FRU: Fructose; GAL: Galactose; MAN: Mannose; APS: Ammonium persulphate; AN: Ammonium nitrate; AS: Ammonium sulphate; AC: Ammonium chloride; SN: Sodium nitrate; UR: Urea; PEP: Peptone; TRY: Tryptone; GLY: Glycine, GEL: Gelatin; YE: Yeast extract and BE: Beef extract).

much focus has been given on microbial enzymes compared with enzymes of plant or animal origins, reflecting the availability of an immense array of catalytic activities, promising lofty yields, effortlessness in genetic manipulation, continuous production due to lack of seasonal fluctuations and swift growth of microorganisms on economic fermentation media. There are 250 officially documented species in the filamentous fungal genus *Aspergillus* [19], of which a variety of fungal strains, including *Aspergillus flavus*, *Aspergillus niger* [20], *Aspergillus clavatus* [21], *Aspergillus oryzae* [22], *A. terreus* [13], *Aspergillus awamori* [23] and *Aspergillus fischeri* [24], were reported to secrete significant quantities of extracellular proteases and other enzymes.

Traditionally, both SmF and SSF have been employed for the production of proteases, but SSF has been preferred and demonstrated as comparatively better than SmF, reflecting the varying environmental and nutritional advantages of this method. Recently, attention has been focused on the effective utilization of solid agricultural by-products for the production of industrially imperative enzymes. Consistent with studies aimed at the efficient production of high-valued, low-cost proteases, various economical agro-residues (MoC, NoC, GnoC, BGP, GGP, CVP, WB, BR, PM, FM, BP, AP and OP) were examined for the production of protease. Amongst all substrates, chickling vetch peels (CVP) were the most suitable substrate for the production of protease through LSSC and SSF (499.99 ± 11 U/ml; 5266.8 ± 202.5 U/gds) (Table 1). However, all substrates supported the production of protease. Similarly, rice [25], wheat bran [26] and other agro-industrial by-products were also utilized for the production of protease.

3.2. Culture amendment for enhanced production of protease

3.2.1. Effects of initial medium pH

To optimize the pH of the fermentation medium for the enhanced production of protease from *A. terreus* NCFT 4692.10, the initial medium pH ranging from 3.0 to 10.0 was adjusted and fermentation was performed. The results showed that pH 5.0 was optimal for the production of this enzyme. The pH positively influenced biomass production in case of protease, whereas the synthesis of enzymes was reduced with increasing pH (Fig. 1a). However, the protease was produced at all pH values, indicating that protease production is pH stable. The final pH of the medium has drastically changed. In addition, the final pH of the medium remained the same

with respect to initial pH 6.0. Hence, the secreted enzyme was an acidic protease. *A. niger* produced proteases at all pH ranges (4.4–9.0), after which the production ceased, consistent with the findings of previous studies [20]. Similar studies on the influence of pH on protease production have been conducted in *A. terrus* between pH 5.5 and 9.5 [27]. Enzyme secretion from microbial strains strongly depends on the extracellular pH, as the culture pH strongly influences many enzymatic reactions.

3.2.2. Effect of temperature

LSSC was performed at 24–45 °C (at an interval of 3 °C) using 1×10^7 spores as inoculum for 96 h to evaluate the effect of incubation temperatures on the generation of mycelial biomass and the production of protease. The temperature strongly influences enzyme and biomass production. The optimum temperature for the production of protease was 30 °C (Fig. 1a). Temperatures of 30 °C, 35 °C and 37 °C have been determined as optimal for protease production in cases of *Aspergillus saitoi*, *Aspergillus tamari, Aspergillus nidulans* and *A. awamori*, respectively [28,23]. Nevertheless, the assessment of enzyme activities in numerous microorganisms is complicated, although discrete culture parameters and enzyme activity determinations have been employed [29].

3.2.3. Effect of fermentation duration on production of protease

At optimized pH and temperature conditions, LSSC was performed at varying fermentation durations for the production of protease. The production of protease was initiated at 24 h and increased for up to 96 h, displaying a gradual decline thereafter. The biomass was substantially increased after 24 h and continued for up to 144 h, with maximum enzyme production at 96 h that subsequently remained stable before showing a decline in the growth profile (Fig. 1b). The effect of the incubation period on protease production in *A. terreus* was consistent with the results of Negi and Banerjee [23] using *A. awamori*. Chimata et al. [30] observed the production of maximum protease at 120 h from a newly isolated *Aspergillus* species when wheat bran, rice bran and green gram husk were employed as substrates. Paranthaman et al. [31] reported that from *A. niger*, maximum protease production was observed after incubation for 72 h in SSF using rice bran as the substrate, which differs from the present study. These differences might reflect the dependency on factors regulating the growth and production of enzymes in the medium. The optimization of the incubation

Table 2
Effect of amino acids, metal ions, vitamins, growth regulators and mixed substrates on mycelia growth and protease production in *A. terreus* NCFT 4269.10[a].

Different sources	Biomass (g/50 ml)	Protease activity (U/ml)	pH*
Amino acids (5 mM/100 ml)			
Alanine	0.13 ± 0.029c	594.6 ± 31c	6.5
Proline	0.24 ± 0.013b	353.1 ± 98g	7.3
Valine	0.14 ± 0.030c	584.9 ± 79c	4.2
Aspartic acid	0.21 ± 0.005b	377.4 ± 63g	6.4
Methionine	0.19 ± 0.015b	532.7 ± 115cd	7.1
Glutamate	0.18 ± 0.005c	356.8 ± 65g	7.0
L-lysine	0.15 ± 0.018c	415.0 ± 113e	6.8
Cysteine	0.21 ± 0.006b	679.6 ± 82ab	6.9
Histidine	0.18 ± 0.007b	658.9 ± 50b	6.8
Phenyl alanine	0.16 ± 0.034bc	618.9 ± 99b	6.8
Isoleucine	0.17 ± 0.014b	512.1 ± 75cd	6.8
Threonine	0.18 ± 0.029b	478.1 ± 34d	7.0
Tryptophan	0.16 ± 0.027bc	399.2 ± 70ef	7.1
Arginine	0.17 ± 0.002bc	367.7 ± 46g	6.9
Leucine	0.17 ± 0.009bc	737.8 ± 130a	6.9
Glycine	0.20 ± 0.010b	436.9 ± 79de	7.0
Serine	0.22 ± 0.010b	462.4 ± 111d	6.9
Metal ions (1 mM)			
Zn^{++}	0.74 ± 0.006a	487.8 ± 11d	5.5
K^+	0.73 ± 0.015a	548.5 ± 13c	5.6
Ag^{++}	0.08 ± 0.009de	442.9 ± 15d	6.9
Fe^{++}	0.08 ± 0.001de	563.1 ± 34c	3.9
Mg^+	0.68 ± 0.038a	439.3 ± 20d	3.9
Cu^{++}	0.08 ± 0.002de	282.7 ± 46h	6.6
Mn^+	0.07 ± 0.001e	390.8 ± 14f	4.3
Ca^+	0.73 ± 0.015a	415.0 ± 34de	9.6
Hg^+	0.10 ± 0.001d	415.0 ± 14de	6.3
EDTA	0.15 ± 0.025cd	400.5 ± 27ef	3.7
Vitamins (mg/100 ml)			
Vitamin C			
10	1.10 ± 0.007c	1150.4 ± 41.2b	6.9
20	1.15 ± 0.086ab	1203.8 ± 102b	6.4
30	1.01 ± 0.002ef	969.3 ± 156.5d	6.1
40	0.54 ± 0.062g	777.8 ± 87.1fg	6.4
50	1.05 ± 0.198d	1035.1 ± 132.1c	6.5
Riboflavin			
10	1.23 ± 0.004a	1507.0 ± 82.1a	6.2
20	0.98 ± 0.003f	912.8 ± 237.2d	6.6
30	1.11 ± 0.003bd	1090.9 ± 12.0c	6.6
40	1.05 ± 0.062d	946.5 ± 106.4de	6.7
50	1.26 ± 0.078a	1134.7 ± 66.9b	6.2
Folic acid			
10	1.12 ± 0.015c	978.1 ± 106.4d	5.2
20	1.20 ± 0.000a	1173.9 ± 228.9b	5.1
30	1.10 ± 0.001cd	939.3 ± 96.1de	5.4
40	0.97 ± 0.023f	674.7 ± 27.4g	5.3
50	0.96 ± 0.008ef	917.4 ± 137.3de	5.5
Vitamin E			
10	0.98 ± 0.002f	898.0 ± 192.2f	6.3
20	1.20 ± 0.001a	1145.6 ± 20.5b	6.2
30	1.13 ± 0.034b	1066.7 ± 176.7c	5.7
40	1.16 ± 0.190ab	1480.6 ± 75.5a	6.3
50	1.22 ± 0.034a	1197.7 ± 87.5b	6.4
*Control (for all above)	0.211 ± 0.006b	499.99 ± 11d	6.5

Table 2 (*Continued*)

Mixed substrates[b]	Ratio			
	1:9	–	10,788.4 ± 772.3c	–
	3:7	–	11,698.6 ± 1167c	–
Chickling pea:horse gram peels	5:5	–	12,657.3 ± 429.1b	–
	7:3	–	6977.8 ± 703.6g	–
	9:1	–	9951.1 ± 446.2d	–
	1:9	–	8506.9 ± 566.3e	–
	3:7	–	14,174.2 ± 1029.6a	–
Chickling pea:pigeon pea peels	5:5	–	12,560.2 ± 1149.8b	–
	7:3	–	8531.2 ± 429.0e	–
	9:1	–	8106.4 ± 274.5f	–
	1:9	–	9635.5 ± 549.2d	–
	3:7	–	12,050.5 ± 909.6bc	–
Horse gram:Red gram peels	5:5	–	7972.9 ± 1424.4e	–
	7:3	–	11,203.4 ± 720.8ab	–
	9:1	–	7087.1 ± 995.3fg	–
**Control	–	–	5269.9 ± 287.5h	–

[a] The liquid static surface culture experiments were performed for four days at 30 °C for all of the cases. *pH: initial pH was adjusted to 7.0 required for the production of protease. Similarly, *Control (for all above) refers to the PM added fermentation broth medium (10%, w/v) devoid of chemicals. The data represent the means ± SD of replicates ($n = 3$). The mean values within a column with different letters are significantly different at $p \leq 0.05$.

[b] **Control refers to only CVP taken as solid-state fermentation medium (10%, w/v). The solid-state fermentation setups were performed as per the above combination for a period of 96 h at 30 °C. The data represent the means ± SD of replicates ($n = 3$). The mean values within a column with different letters are significantly different at $p \leq 0.05$.

regimen is an obligatory stride in making the production of industrial enzymes economically feasible.

3.2.4. Effect of carbon sources on the production of protease

Amongst all carbon sources supplemented with the CVP fermentation broth, none has participated in enhancement of protease production compared with the control (Fig. 1c). Negi and Banerjee [23] reported that the supplementation of lactose as a carbon source enhanced protease production. There are several reports on the utilization of diverse carbon sources and strain type that differentially influence the production of this extracellular enzyme [32].

3.2.5. Influence of nitrogen sources on protease production

Different organic and inorganic nitrogen sources were individually supplemented into the fermentation medium and evaluated for the enhanced production of protease. The supplementation of peptone only favoured the highest protease production amongst all organic and inorganic nitrogen sources evaluated (Fig. 1c). Amongst the organic nitrogen substrates utilized, peptone supported protease yield, consistent with the results of Akhter et al. [33].

3.2.6. Effect of amino acids on production of protease

In the present study, seventeen amino acids were supplemented into the CVP fermentation medium and the suitability for the production of protease was evaluated. The results showed that leucine at a concentration of 1 mM enhanced ∼1.5-fold synthesis of protease, whereas maximum biomass was attained with proline (Table 2). Similarly, cysteine, histidine and alanine also supported the production of protease.

3.2.7. Effect of metal ions

Amongst the metal ions evaluated, Fe^{2+} significantly affects the enhanced production of protease (Table 2). It was also reported that this protease was activated through Ca^{2+}, Fe^{2+} and Mg^{2+} [34]. The role of metal ions in stimulating thermal stability might reflect the consequences of these molecules on the integrity of the enzyme conformation or alterations in the solvent polarity, ionic potency and balance that might alter the surface pH and/or active site conformation [35].

3.2.8. Effect of vitamins on production of protease

The effect of four vitamins (Vitamin C, riboflavin, folic acid and Vitamin E) on the production of protease was tested at varying concentrations as shown in Table 2. The results of the present study showed that riboflavin at a concentration of 10 mg/100 ml exhibited ∼3-fold

protease activity (1507.05 ± 84.14 U/ml) (Table 2). Afifi et al. [36] also reported that vitamin C had the most pronounced effect on the growth, protein accumulation and amylase production in *P. olsonii*, which validates the demand of vitamin for the production of protease and growth of *A. terreus*.

3.2.9. Effect of plant growth regulators on production of protease

The effect of growth hormones (BAP, 2-4 D, Kinetin and gibberellic acid) on protease production was examined after supplementing with fermentation medium at a concentration of 0.025% (w/v). However, none of these growth regulators support enzyme secretion as compared with the control (data not shown). Negi and Banerjee [23] also examined the effects of growth hormones on amylase and protease production and concluded that indol acetic acid (IAA) and indol-3-butyric acid (IBA) both stimulated the production of the enzymes produced from *A. awamori*. Negi and Banerjee [23] indicated that naphthalene acidic acid (NAA) and 2-4, D enhanced protease production but reduced amylase production, which is not *vis-a-vis* to the findings of *A. terreus*.
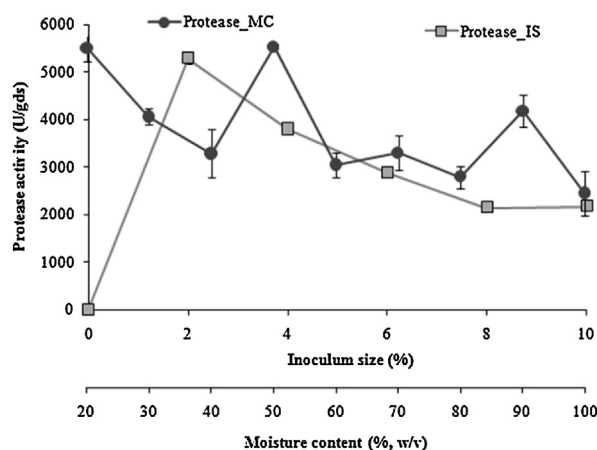

Fig. 2. Effect of inoculum size (■) and initial moisture content (●) on the production of extracellular protease from *A. terreus* NCFT 4269.10 performed for 96 h at 30 °C using solid-state fermentation.

3.2.10. Effect of mixed substrates on enzyme production

The synergistic effect of different combinations of agro-residues was assessed for the enhanced production of protease using the SSF method. The results

Table 3
Effect of soaking time, repeated extraction and various extractants on protease recovery.

Extraction	Protease activity		
	U/gds	U/mg	U/g
Soaking time (h)			
0.5	5121 ± 270d	2938.2 ± 390d	1024.2 ± 28f
1	2985 ± 170h	1693.1 ± 46h	597 ± 34e
3	3361 ± 120g	2084.8 ± 180fg	672.2 ± 24d
5	4345 ± 170f	3723.7 ± 72a	869 ± 34e
7	6019 ± 340b	3422.7 ± 140b	1203.2 ± 68a
24	6444 ± 360a	3978 ± 137a	1288.8 ± 72b
48	5388 ± 140c	2624.9 ± 25d	1077.6 ± 28b
72	5934 ± 90bc	3236.5 ± 130bc	1186.8 ± 18f
96	5121 ± 270d	3285 ± 130c	1024.2 ± 54e
Repeated extraction			
1	5294 ± 120c	1634.2 ± 51h	1058.8 ± 24b
2	4333 ± 220f	1354.3 ± 70j	866.6 ± 44d
3	4591 ± 340e	2338.4 ± 120e	918.2 ± 68cd
Different extractants			
PB (0.1 M)	5321 ± 160c	2119.6 ± 130f	1064.2 ± 32b
PB + Trit × 100 (0.1%, w/v)	3653 ± 120g	2486.9 ± 90e	730.6 ± 24e
PB + Trit × 100 (0.5%, w/v)	2925 ± 90h	1913.6 ± 24h	585 ± 18f
PB + Trit × 100 (1%, w/v)	4915 ± 150e	2736.8 ± 80d	983 ± 30c
PB + Urea (1 M)	2169 ± 90i	2041.7 ± 50g	433.8 ± 18fg
PB + Amm. Sulphate (1 M)	1481 ± 170i	2191.7 ± 390f	296.2 ± 34h
NaCl (0.5%)	1517 ± 190j	1540.2 ± 140i	303.4 ± 38h
DW (MilliQ)	6311 ± 200a	1704.8 ± 110h	1262.2 ± 40a
Czapek Dox	6165 ± 340a	1336.7 ± 190j	1233 ± 68a

The solid-state fermentation setups were performed for a period of 96 h at 30 °C. ND: not determined. The data represent the means ± SD of replicates (n = 3). The mean values within a column with different letters are significantly different at $p \leq 0.05$.

Table 4
Purification summary of isolated protease.

Steps	Volume	Activity (U/ml)	Total protease activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude	400	499.99	199,996	923.33	219.603	1.0	100
Ammonium sulphate precipitation	12	658.69	7904.28	35.08	225.321	1.02	3.95
Sephadex G-100	5	1231.86	6159.3	17.9	344.1	1.6	3.08

showed that CVP:Pigeon pea peels at a ratio of 3:7 and CVP:horse gram peels at a ratio of 5:5 exhibited the highest protease production (Table 2). Similarly, Chutmanop et al. [37] have reported that rice bran has reduced porosity for agreeable solid-state fermentation and could be more effective only when employed in combination with wheat bran for synthesis of protease from *A. oryzae*.

3.2.11. Effect of inoculum size

Effect of inoculum size on protease production revealed that a 2% (w/v) inoculum size was optimum for the production of protease (Fig. 2). In all cases, a further increase in the inoculum size resulted in decreased enzyme synthesis. Wu et al. [38] suggested that a 5% inoculum level leads to the enhanced production of protease using *A. terreus*, whereas, in this present study, an inoculum size of 2% was suitable for the synthesis of protease. A further increase in inoculum size resulted in decreased enzyme synthesis. Studies have also shown that there was a decline in any production with increased

inoculum size was associated with high biomass, which is responsible for the production in enzyme production under nutrient scarce conditions in fermentation mashes.

3.2.12. Effect of initial moisture content

As moisture content plays an important role in SSF and greatly affects the production; hence, the moisture content of the substrate was adjusted and fermentation was performed. A moisture content of 50% (w/w) was optimum for the production of protease (Fig. 2). A further increase in moisture content generated in a gradual decrease in protease activity. Similarly, 50% initial moisture content was optimal for protease production from *P. godlewskii* SBSS 25, indicating that the water-binding capacity of chickling vetch peels was low. High moisture content was not conducive for the mycelial growth of *P. godlewskii* SBSS 25, reflecting the agglomeration of substrate particles and thereby, reducing the oxygen level [39].
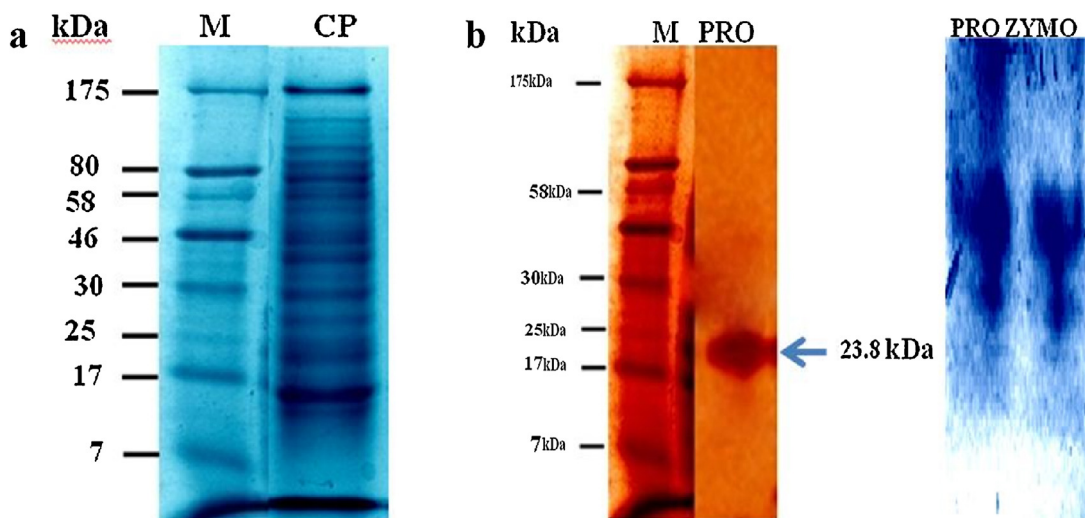

Fig. 3. Electrophoretic analysis of protease (a) (M: Marker; CP: Crude protease); (b): (M: Marker; PRO: Purified protease); (PROZYMO: Zymographic analysis of protease).

3.3. Down streaming of protease

For the efficient and enhanced recovery of protease from solid fermented medium, different extractants and soaking times were examined. The results showed that soaking for 24 h at first extraction with MilliQ water was superior for the maximum recovery of protease compared with other extractants (Table 3). Aikat and Bhattacharyya [26] reported that 0.6 M phosphate buffer (pH 8.0) was optimal for the recovery of protease from *Rhizopus oryzae* using wheat bran as the substrate with 72 h of soaking time at the first extraction. These authors also reported that Czapek Dox salt solution was better than distilled water. However, distilled water was more efficient in enzyme recovery compared with Czapek Dox solution, suggesting that different solvents can be used for the down streaming of enzymes. Until recently, the mechanism behind this hypothesis has not been clearly understood.

3.4. Purification of protease

The cell-free dialyzed protease displayed 7904.28 U with a specific activity of 225.321 U mg^{-1} protein, representing 3.95% yield and 1.02-fold purification. The partially purified protease (dialyzed ammonium sulphate-precipitated fraction) was further fractionated through a column of Sephadex G100 and the maximum amount of protease, representing 1.6-fold purification (Table 4). The protease was eventually purified to 1.6-fold homogeneity, 3.08% yield and a specific activity of 344.1 U/mg using ammonium sulphate precipitation followed by Sephadex G-100 gel filtration chromatography (supplementary material). Similarly, Benazir et al. [32] attained approximately 1.17-fold purification with a protease yield of 82.47% isolated from *A. niger*. Enzymes from *A. oryzae* KSK-3 were also concentrated through ultrafiltration and sequentially purified using ammonium sulphate precipitation and ion exchange and gel filtration chromatography according to Shirasaka et al. [25].

3.5. Molecular weight determination through SDS-PAGE and zymographic analysis

Both crude and purified proteases were subjected to protein profiling and molecular weight determination. The crude protease displayed 13 bands within the range of 7–175 kDa (Fig. 3), whereas the purified protease displayed a single band with molecular weight of 23.8 kDa (Fig. 3). When the zymogram was prepared using casein, only a single hydrolyzed band was

Table 5
Fermentation kinetics of produced protease.

Optimum conditions	$Y_{c/s}$ (U/g)	$Y_{x/s}$ (g/g)	α (U/g)	β (U/g/h)	q_c (mg/g/h)	dx/dt (g/L/h)	dP/dt (U/ml/h)	x (mg ml^{-1})	μ_{max} (mg/L/h)
pH (5.0)	91.72	0.015	5879.48	0.955	3.250	0.016	4.77	1.56	16.25
Temperature (30 °C)	100.80	0.088	1140.27	1.050	18.416	0.092	5.25	8.84	92.08
Incubation period (96 h)	104.74	0.047	2219.06	1.091	9.833	0.049	5.45	4.72	49.16
Carbon source (Maltose)	88.00	–	–	0.916	–	–	4.58	–	–
Organic nitrogen (Peptone)	96.06	–	–	1.001	–	–	5.00	–	–
Inorganic nitrogen (NH$_4$S$_2$O$_8$)	91.46	–	–	0.952	–	–	4.76	–	–
Amino acid (Leucine)	139.90	0.042	3236.09	1.416	8.753	0.044	7.08	4.20	43.74
Metal ion (Fe^{2+})	112.6	0.016	6702.38	1.172	3.500	0.017	5.86	19.74	17.50
Vitamins (Riboflavin, 10 mg)	301.4	0.246	1224.20	3.139	51.296	0.256	15.69	24.62	256.45
Inoculums size (2%), SSF	5293.5	–	–	55.14	–	–	–	–	–
Moisture content (50%), SSF	5837.1	–	–	60.81	–	–	–	–	–

observed. Analyses through SDS-PAGE and zymography for the production of protease from different fungal species were performed. Variations in molecular weights have also been reported for different species, such as *A. niger* (60 kDa; [32]) and *A. oryzae* KSK-3 (30 kDa; [25]).

3.6. Fermentation kinetics study

The data obtained from the above experiment was subjected to kinetics analysis for the calculations of $\mu_{\max}$ (h^{-1}) (specific growth rate), q_p (unit product produced/g spores/h), q_s (g substrate consumed/g spores/h), $Y_{p/s}$ (unit product produced/g substrate consumed), $Y_{p/x}$ (unit product produced/g spores formed), $Y_{x/s}$ (g spores/g substrate utilized), Q_p (g spores produced/l/h) and Q_s (g substrate consumed/l/h). The kinetics evaluation also revealed that the optimum fermentation period for the extracellular production of protease from *A. terreus* was 96 h with a constant growth rate. Nonetheless, growth and enzyme secretion were significantly affected after the engineering of fermentation media (supplementation of nutrients) and optimization of different parameters. The specific production rate and growth coefficient revealed hyperproducibility of the extracellular protease (Table 5). Iftikhar et al. also reported similar observations for the production of protease from *Rhizopus oligosporus* var. *microsporus* [40].

4. Conclusion

This study revealed the possibilities of the effective utilization of agro-wastes in fermentation (LSSC and SSF) processes as potential substrates that act as carbon and nitrogen sources and ultimately produced industrially relevant enzymes. However, the production of protease will be efficient using CVP as one of the medium components through both LSSC and SSF in *A. terreus*. Obviously, such amalgamation of processes would not only facilitate the trimming of the entire production cost but also focus on the approaches towards the effective management of agro-wastes. This study portrays a gainful, expedient, non-tedious, relatively easy technique for scaling up the enhanced production of protease.

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