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Enhanced production of alkaline protease by *Neocosmospora* sp. N1 using custard apple seed powder as inducer and its application for stain removal and dehairing

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ABSTRACT

Alkaline proteases find extensive applications ranging from detergent industries to therapeutics due to their broad alkaline range activity. The present work describes the first report on optimization of alkaline protease production from *Neocosmospora* sp. N1 and explores its utility in detergent and leather industries. Wheat bran was found to be a suitable substrate for protease production under solid state fermentation amongst several low cost agro-based materials used in this study. Protease production was enhanced when wheat bran was supplemented with a novel inducer, custard apple seed powder in the ratio of 4:1 which increased enzyme production up to 1.88 fold. One factor at a time approach was used to select parameters important for production and response surface methodology was used for further optimization. The optimum level was attained at 123 h fermentation time, 63% moisture content, 1×10^8 spores/ml inoculum size and 1.4 mm particle size which resulted in 3.12 fold increase in protease production. The partially purified enzyme exhibited maximum activity at 60 °C and was active over a wide pH range of 8–12. Protease was compatible with various laundry detergents *viz.* Tide, Surf Excel, Ariel, Wheel etc. showing more than 80% stability even after 3 h of incubation and was efficient in removing blood stain from the cotton cloth. The enzyme was also more efficient in dehairing goat skin as compared to conventional leather processing treatment.

1. Introduction

Protease enzymes are ubiquitous in nature catalysing hydrolysis of protein molecules into peptides and amino acids (Sumantha et al., 2006). In fact, proteases make up the largest single family of enzymes engrossing several bioengineering and biotechnological applications. Alkaline proteases from the array of proteases, account for almost 40% of the total worldwide sales of enzymes owing to their activity and stability under harsh operational conditions (Wahab and Ahmed, 2018). Ever since the advent of biotechnology, alkaline proteases, primarily subtilisin derived from *Bacillus* species have served as an essential ingredient of modern laundry detergents (Maurer, 2004). The performance of a high grade detergent protease depends upon several parameters such as degradation of protein stain, compatibility with detergent components like surfactants, complex agents, perfumes and other enzymes, stability in the presence of oxidizing agents such as bleach and shelf life in detergent formulations (Vojcic et al., 2015). Advances in improving performance of detergent proteases are ongoing in terms of cost effectiveness, superior washing performance with fabric care and improved stability. Hence, spotlight on subtilisin like proteases derived from fungal species having robust properties have gained much interest in recent years. Fungi are known to secrete extracellular enzymes in large amounts and are a preferred choice for enzyme production due to ease in downstream processing. Recently, alkaline proteases have also gained prime importance in leather industries for their ability to dehair animal skin in a safe and eco-friendly manner (Sujitha et al., 2018). The foremost step in conventional leather processing makes use of lime and sulphide for removing hairs from animal skin. This process discharges considerable amount of harmful effluent causing severe impact on water and soil. Additionally, the inability to have precise control over the chemical reaction can cause skin damage and loss of hair and wool (Khandelwal et al., 2015). Therefore, proteases may serve as a greener alternative, replacing the use of toxic chemicals in leather

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Abbreviations

WB	Wheat bran
CASP	Custard apple seed powder
SSF	Solid state fermentation
BBD	Box-Behnken Design

industries.

The cost of enzyme plays a critical role, delimiting the use of alkaline proteases for various applications. A large part of this cost is accounted by the production media and steps involved in the downstream processing of the enzyme (Meena et al., 2013). Recent years have witnessed new and innovative biotechnological processes for production of proteases involving solid state fermentation (SSF) as a promising technology. SSF mimics the natural habitat of microorganisms utilizing agro-industrial wastes as solid substrates for supporting the growth and metabolism of the microorganisms (Pandey, 2003). In addition, a number of physio-chemical factors affect the growth of microorganisms like temperature, pH, aeration, water activity, bed properties, nature of solid substrate and its particle size (Thomas et al., 2013). Response surface methodology proved to be a valuable tool for studying the effect of several factors on enzyme production by varying them simultaneously in a limited number of experiments, thus saving time and cost of optimising production processes. Each microorganism is unique in itself having distinctive requirements for growth and production of extracellular enzymes, hence the criteria for achieving optimised production varies markedly.

SSF has proved to be more economic technology as compared to submerged process especially in countries having abundant biomass and agro-industrial waste available all year round. Moreover, it offers several advantages over submerged process including high volumetric productivity, relatively high concentration of products, less effluent generation and requires simple fermentation equipments (Pandey et al., 1999; Patidar et al., 2016). Over the years, fungi remain a preferred choice while using SSF for enzyme production. Till date, several reports on optimization of protease production in submerged fermentation have been published. In a study, Mechri et al. (2017) optimised cultural parameters for alkaline protease production in submerged fermentation from *Lysinibacillus fusiformis*. Similarly, Mishra (2016) optimised production using *Brevibacillus brevis* and Sathishkumar et al. (2015) reported optimization of production using *Bacillus subtilis* for alkaline protease in submerged fermentation. Several fungal species like *Aspergillus* (de Castro et al., 2015a), *Penicillium* (Agrawal et al., 2004), *Fusarium* (Ali and Vidhale, 2013) have been exploited for alkaline protease production in SSF. To the best of authors' knowledge, this is the first report on optimization of alkaline protease production using *Neocosmospora* species.

Enzymes required for various industrial purposes are sought on the basis of their characteristic properties like stability at specific temperature and pH, activity in the presence of metal ions, organic solvents, surfactants etc. Hence, novel microorganisms producing enzyme with attractive properties are explored. Recently, filamentous fungi producing alkaline protease have gained much attention due to their rich biochemical diversity, ease in growth on inexpensive medium and cost effective purification of enzyme from the medium (Abidi et al., 2008). Thus, the present work describes the optimization of production of alkaline protease from a newly discovered Ascomycete, *Neocosmospora* sp. N1 isolated from soil. The composition of media significantly affects the cost of production and extracellular protease synthesis in microorganisms. Hence, agro-based material, wheat bran was selected as substrate for solid state fermentation and custard apple seed powder was used as a novel inducer in the system to enhance protease production. Further, the production parameters were standardised using OFAT approach and statistically optimised by response surface methodology using Box-Behnken Design (BBD) to achieve maximum level of alkaline protease production. The partially purified enzyme was thermostable and active at alkaline pH. The enzyme was explored to check its effectiveness in industrial applications like stain removal and dehairing of skin. The exceptional properties of protease from *Neocosmospora* sp. N1 studied in an earlier report (Matkawala et al., 2019) allow researchers to explore more insights about the enzyme and utilize it commercially.

2. Materials and methods

2.1. Microorganism

The present study delineates alkaline protease production using a novel fungus isolated from soil of a local fruit market in Indore, MP, India. Molecular identification of the strain was carried out by sequencing 18S rRNA region. Sequence data was aligned with publicly available sequences and submitted to GenBank under accession number **MK417797**. The isolated strain was designated as *Neocosmospora* sp. N1. The fungus was periodically sub cultured and maintained on potato dextrose agar slants.

2.2. Inoculum preparation

Inoculum was prepared by harvesting spores from a 5 day old fungal culture using sterile distilled water, filtered through sterile glass wool and counted using a counting chamber. Spore suspension of 1×10^7spores/ml was used for inoculation.

2.3. Selection of solid substrate

Agro-industrial residues namely, wheat bran, soy bran, corn bran, rice bran, orange peel, papaya peel, soybean husk, wheat husk, peanut shell were screened for alkaline protease production under SSF. All the substrates were dried and sieved to obtain particle size of 0.5–1 mm. SSF was carried out by taking 10 gm dried substrate in 250 ml Erlenmeyer flask, moistened with 5 ml distilled water (50% v/w) having pH 8. All the flasks were autoclaved at 121 °C, 15 lbs for 20 min. 1 ml of spore suspension was inoculated and the flasks were incubated at 30 °C for 96 h. Enzyme assay was performed in triplicate.

2.4. Effect of custard apple seed powder

The effect of CASP on enzyme production was studied by combining wheat bran (WB) and CASP in different ratio (5:1, 4:1, 3:1, 2:1, 1:1, 1:0, 0:1, 1:2, 1:3, 1:4 and 1:5) and carrying out the fermentation process.

2.5. Enzyme extraction

The fermented medium was thoroughly mixed with 50 mM Tris-HCl buffer, pH 8.5 (1:20 dilution, w/v) and incubated at 30 °C on a shaker at 120 rpm for 1 h. The slurry was centrifuged at 10,000×*g* for 15 min at 4 °C. The supernatant obtained was used as crude enzyme extract.

2.6. Determination of alkaline protease activity

Alkaline protease activity was determined using alkali soluble casein as substrate according to the method of Charles et al. (2008) with slight modifications. The reaction mixture consisted of 1 ml of 2% (w/v) casein prepared in Tris-HCl buffer, pH 8.5 with 0.1 ml of the enzyme and incubated for 30 min at 60 °C. The reaction was stopped by adding 2 ml of 0.4 M TCA to precipitate undigested protein. The tubes were centrifuged at 5000×*g* for 5 min to remove all the undigested proteins. A 0.5 ml aliquot from the supernatant was withdrawn and mixed with 5 ml of 0.4 M Na_2CO_3. In this tube, 1 ml of 0.5 N Folin Ciocalteau reagent was added to visualise the reaction. Absorbance was read at 660 nm using

UV–visible spectrophotometer. One unit of enzyme activity (U) was defined as the amount of enzyme required to liberate 1 μmole of tyrosine equivalent per min under standard assay conditions. All the experiments were performed in triplicate and standard error was calculated.

2.7. Scanning Electron Microscopy

Detailed structural analysis of uninoculated substrate and the fermented medium was performed by drying the samples and analysing them by Scanning Electron Microscopy (SEM) type JEOL JSM 5600 at UGC DAE Consortium, Indore.

2.8. Chemical composition of substrates

Carbohydrate, protein, lipid, moisture and ash contents of WB and CASP were determined according to the methods described by the Association of Official Analytical Chemists (AOAC, 2010). All tests were performed in triplicate and results were expressed as mean ± standard deviation.

2.9. Statistical optimization of process parameters

Process parameters for alkaline protease production were studied by one factor at a time (OFAT) approach and four variables namely; fermentation time, inoculum size, moisture content and particle size of CASP were identified to play a significant role in the fermentation process. The optimum levels and interactions between significant parameters were investigated by response surface methodology using Box-Behnken Design, (Box and Behnken, 1960). Design-Expert 11.0 software (Stat-Ease Inc., Minneapolis, MN, USA) was used to setup experimental range and levels of the four independent variables used in Box-Behnken Design are given in Table 1. The number of experiments (N) required for the development of BBD was defined by the following equation:

$$N = 2k\ (k\text{-}1) + C_O$$

Where, k is the number of variables and C_O is the number of central points.

This equation was used to develop mathematical correlation between four variables on the production of alkaline protease by experimentally performing 29 runs with five replicates at central point. The response data was used to fit the following quadratic polynomial model equation:

$$Y = \beta_0 + \Sigma\ \beta_i\ X_i + \Sigma\ \beta_{ii}\ X_i^2 + \Sigma\ \beta_{ij}\ X_{ij}$$

Where, Y represents predicted response; X_i represents independent variables; X_{ij} are variables interacting with each other; β_0 is the coefficient of fitted response; β_i is linear coefficient; β_{ii} is quadratic coefficient and β_{ij} represents interaction coefficient.

Table 2 represents the experimental design of BBD in coded levels of the four variables. Temperature 30 °C and pH 8 were maintained for all the experimental runs. Flasks were analysed for alkaline protease activity at specific time intervals of 48, 96 and 144 h as planned in BBD.

Table 1
Levels of independent variables used in the experimental design.

Variable	Name of factor	Unit	Range and Levels		
			−1	0	+1
A	Fermentation time	h	48	96	144
B	Moisture content	% (v/w)	30	50	70
C	Inoculum size	$1 \times 10^{\times}$ spores/ml	6	7	8
D	Particle size of CASP	mm	0.5	1	1.5

2.10. Validation of the experimental model

The validation of model equation was conducted in triplicates for alkaline protease production under optimised conditions and the results obtained empirically were compared with the response predicted by the model. Growth kinetics and protease production in the optimised medium were also determined. The fungal growth in the medium was estimated by measuring *N*-acetyl-D-glucosamine (NAGA) contents according to the method described by Sakurai et al. (1977).

2.11. Partial purification and characterization of protease

The optimised medium was used for alkaline protease production in SSF. The crude extract obtained (described under 2.5) was subjected to ammonium sulphate precipitation at 0–90% saturation and placed for overnight incubation at 0–4 °C. The protein precipitate was collected by centrifugation at 9000×*g* for 15 min and was re-suspended in 25 mM Tris-HCl buffer, pH 8.5. Desalting was performed using Sephadex G-25 column chromatography using the same buffer. Optimum temperature of the enzyme was determined by calculating enzyme activity in the range of 10–90 °C and pH optima of the enzyme was determined by measuring enzyme activity in the pH range of 6–12 using casein as a substrate.

2.12. Evaluation of washing performance of protease

2.12.1. Compatibility and stability with laundry detergents

The stability and compatibility of the protease was studied using various commercial detergents *viz*. Surf Excel (Hindustan Unilever Limited), Ariel (Procter & Gamble), Tide (Procter & Gamble), Wheel (Hindustan Unilever Limited) and other locally available detergents *viz*. Chameli (Burhani Enterprises, Indore) and Badshah (Saify Hardware Stores, Indore). These detergents were diluted in water at the final concentration of 7 mg/ml. The endogenous enzymes present in commercial laundry detergents were inactivated by boiling at 100 °C for 15 min. The partially purified protease was mixed with detergent solution in a ratio 1:1 (v/v) and incubated at 40 °C for 3 h (Wahab and Ahmed, 2018). Aliquots were withdrawn after every 30 min time intervals and residual enzyme activity was measured. The crude enzyme activity of a control sample (without any detergent), incubated under similar conditions, was taken as 100%.

2.12.2. Blood stain removal

The stain removal ability of the protease was evaluated using 4 cm × 4 cm pieces of white cotton cloth. The cloth pieces were evenly stained with 0.5 ml of animal blood followed by air drying for 18 h and subsequently washing with distilled water to remove excess blood. These cloth pieces were again air dried and subjected to different wash treatments. A stained cloth piece was treated with a local laundry detergent devoid of any endogenous enzyme. Another piece was treated with the protease used in this study. Finally, one of the stained cloth piece was washed with a mixture of detergent and protease. All the above washing treatments were given at 40 °C for 15 min followed by air drying. The cloth pieces were visually analysed for checking the efficiency of stain removal. Untreated stained cloth piece was considered as control.

2.12.3. Fourier-transform infrared spectroscopic analysis

The structural changes on the cotton cloth fibres after stain removal by enzyme and detergent washing treatments were analysed using Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy (ATR-FTIR) at School of Chemical Sciences, Devi Ahilya University, Indore. The spectra were performed using a Frontier PerkinElmer FTIR-SP10 spectrophotometer with a resolution of 4 cm^{-1} and scanning a wavelength range from 500 to 4000 cm^{-1}. For each sample, the diamond crystal of an ATR apparatus was used and the applied torque was

Table 2
Experimental design and results for optimization of process parameters for alkaline protease production using Box-Behnken Method.

Std	Run	Fermentation Time (h)		Moisture Content% (v/w)		Inoculum Size 1×10^X		Particle Size (mm)		Observed Response (U/ml)	Predicted Response (U/ml)
		Coded	Decoded	Coded	Decoded	Coded	Decoded	Coded	Decoded		
11	1	−1	48	0	50	0	7	1	1.5	223.1	217.7
7	2	0	96	0	50	−1	6	1	1.5	224.3	231.14
20	3	1	144	0	50	1	8	0	1	357.2	349.85
15	4	0	96	−1	30	1	8	0	1	87.3	93.48
14	5	0	96	1	70	−1	6	0	1	254.6	151.11
8	6	0	96	0	50	1	8	1	1.5	307.12	309.95
3	7	−1	48	1	70	0	7	0	1	201	199.70
26	8	0	96	0	50	0	7	0	1	280.4	285.99
28	9	0	96	0	50	0	7	0	1	283.1	284.62
2	10	1	144	−1	30	0	7	0	1	75.2	74.06
17	11	−1	48	0	50	−1	6	0	1	239.5	246.53
18	12	1	144	0	50	−1	6	0	1	221.65	217.43
4	13	1	144	1	70	0	7	0	1	315.3	312.63
24	14	0	96	1	70	0	7	1	1.5	319	315.23
19	15	−1	48	0	50	1	8	0	1	181.6	185.05
29	16	0	96	0	50	0	7	0	1	284.2	282.43
23	17	0	96	−1	30	0	7	1	1.5	53.1	45.26
9	18	−1	48	0	50	0	7	−1	0.5	198	193.4
6	19	0	96	0	50	1	8	−1	0.5	284.5	275.27
22	20	0	96	1	70	0	7	−1	0.5	239.5	241.65
21	21	0	96	−1	30	0	7	−1	0.5	126.9	130.34
1	22	−1	48	−1	30	0	7	0	1	51.4	51.68
13	23	0	96	−1	30	−1	6	0	1	104	103.04
25	24	0	96	0	50	0	7	0	1	285.88	285.99
12	25	1	144	0	50	0	7	1	1.5	245.3	252.59
16	26	0	96	1	70	1	8	0	1	328.4	232.05
10	27	1	144	0	50	0	7	−1	0.5	285.7	293.77
5	28	0	96	0	50	−1	6	−1	0.5	287.9	282.68
27	29	0	96	0	50	0	7	0	1	296.4	285.99

kept constant to maintain uniform pressure.

2.13. Dehairing studies

2.13.1. Hair removal from goat skin

Dehairing property of the crude enzyme was studied using fresh goat skin pieces (4 × 4 cm) with hair. Enzymatic treatment was performed by soaking skin piece in 50 mM Tris-HCl buffer, pH 8.5 containing 500 U/ml of enzyme and incubating at 40 °C in a water bath for 24 h. A skin piece soaked in the same buffer and incubated under similar conditions was considered as control. Chemical hair removal was carried out by soaking the skin in a mixture of 6% lime and 3.5% sodium sulphide incubated under the same conditions. After incubation, hairs were gently scratched from the surface of skin pieces using a spatula. The hair and skin pieces obtained from chemical and enzymatic treatments were gradually dehydrated and analysed by SEM.

2.13.2. Histological examination of dehaired skin

Dehaired pelts obtained from the enzyme and chemical treatment on goat skin were fixed in 10% formalin solution for 24 h. The fixed tissues were embedded in paraffin blocks and 4 μm thin sections were cut using microtome. These sections were stained using hematoxylin-eosin stain and observed under light microscope for histological examination (Rao et al., 2009).

3. Results and discussion

3.1. Selection of substrates for alkaline protease production

This study aimed to screen several agro-based materials for production of alkaline protease using the Ascomycete, *Neocosmospora* sp. N1. WB was found to be a superior solid substrate for alkaline protease production exhibiting 62.4 U/ml activity as compared to other agro-based materials used in this study (Table 3). The use of inexpensive substrates in the production medium is preferred in order to reduce the cost of production and enhance environment sustainability (Show et al., 2015). Therefore, WB was selected as a suitable substrate to carry out the fermentation process. The production of enzymes in SSF is governed by the composition of substrates and process parameters (de Castro et al., 2015a). Selecting a support material which can also supply nutrients in the medium is a characteristic attribute influencing the growth of microorganism and formation of desired product in SSF (Trivedi et al., 2012). Several studies have been reported illustrating the use of WB as a powerful solid material for production of alkaline protease (Mechri et al., 2017; Meena et al., 2013). Furthermore, WB has been employed as a universal substrate for production of many industrially important enzymes like cellulase and xylanase acting as a complete nutritious feed for microorganisms (Limkar et al., 2019).

Table 3
Screening of solid substrates for alkaline protease production by *Neocosmospora* sp. N1 in solid state fermentation.

Solid Substrate	Enzyme Activity (U/ml)
Wheat bran	62.4 ± 2.80
Corn bran	34.46 ± 1.87
Soy bran	32.86 ± 1.72
Rice bran	15.17 ± 0.73
Soybean husk	12.45 ± 0.64
Wheat husk	35.6 ± 1.76
Peanut shell	8.99 ± 0.41
Orange peel	10.4 ± 0.45
Papaya Peel	12.5 ± 0.61

It is well established in literature that the production of extracellular proteases by microorganisms can be induced by adding complex protein in the medium. Also, the requirement of nitrogen varies with the type of microorganism (Kumar and Takagi, 1999). Using this background, WB was combined with CASP (having good amount of protein) in different ratio to study its effect on alkaline protease production (Fig. 1A). Interestingly, a 1.88 fold increase in enzyme production was observed when WB and CASP were combined in the ratio of 4:1. Fig. 1B shows the

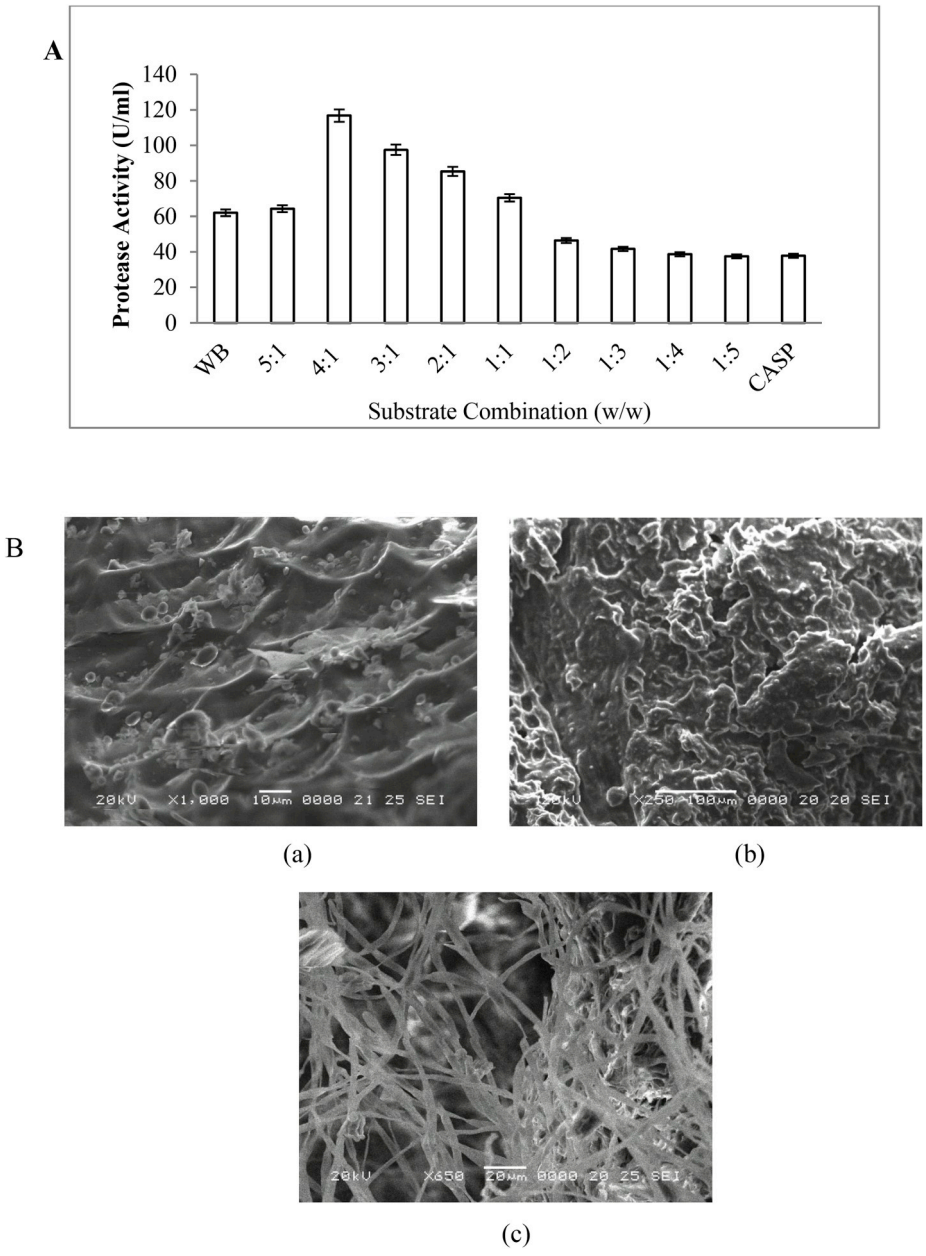


Fig. 1. (A) Effect of CASP on alkaline protease production, when combined with wheat bran (WB) in different ratio. (B) SEM images of (a) dried CASP (1000x) (b) dried wheat bran (250x) (c) growth of *Neocosmospora* sp. N1 on fermented medium (650x).

SEM images of dried substrates and the fermented medium. The microscopic structures of CASP and WB define their porous nature with efficient adsorption capacity. These can be easily approached by the fungus for obtaining nutrients required for the growth and product formation. Scientists have used various materials as inducers for protease production. Bhattacharya et al. (2012) utilised the extract of Mahua flowers to enhance protease production from *Aeromonas* species in submerged fermentation. In another study, Abidi et al. (2008) used *Spirulina* (algae) as an inducer for production of alkaline protease by *Botrytis cinerea* while Agrawal et al. (2004) supplemented WB with soy protein isolate for enhancing alkaline protease production by *Aspergillus oryzae and Beauveria felina*.

Proximate analysis of both the substrates was performed which revealed that WB and CASP comprised of 27.12% and 57.61% protein, respectively. The carbon/nitrogen ratio of substrates as well as their optimised combination was calculated and given in Table 4. The combination of WB: CASP (4:1) has a ratio of 1.58 which was found to be less than the carbon/nitrogen ratio of WB alone, hence it favoured protease production. de Castro et al. (2015b) demonstrated a negative correlation between C/N ratio with protease production and used a quaternary mixture of WB, soybean meal, cotton seed meal and orange peel having a C: N ratio of 2.35 for protease production from *Aspergillus niger*. In another study for acidic protease production by *Aspergillus foetidus*,

Table 4
Proximate analysis of substrates used for production of alkaline protease in SSF.

Chemical components (%)	WB	CASP	WB:CASP (4:1)
Carbohydrate	62.73 ± 3.1	29.23 ± 1.44	59.12 ± 2.43
Protein	27.12 ± 1.2	57.61 ± 2.38	37.43 ± 1.54
Lipid	3.15 ± 0.17	7.06 ± 0.31	1.56 ± 0.056
Moisture	4.78 ± 0.21	2.34 ± 0.11	1.71 ± 0.058
Ash	2.22 ± 0.12	3.76 ± 0.14	1.74 ± 0.062
C:N ratio	2.31 ± 0.13	0.51 ± 0.02	1.58 ± 0.057

Souza et al. (2017) also justified that low C/N ratio of the substrate favours protease production. Hence, the combination of WB: CASP (4:1) was used for further optimization of protease production from *Neocosmospora* sp. N1.

3.2. Statistical optimization of process parameters

The effect of different process parameters on alkaline protease production was studied by OFAT approach, and four factors namely, fermentation time, moisture content, inoculum level and particle size of CASP were found to be significant. Response surface methodology using Box Behnken Design was employed to determine the optimum level of significant variables to enhance alkaline protease production by *Neocosmospora* sp. N1.

A set of 29 experiments was performed and the results of the experiments on effect of four independent variables studied at three levels and five central points are presented in Table 2. The statistical significance of the model equation was evaluated by analysis of variance (ANOVA) using Design-Expert 11.0 software and data is presented in Table 5. The generated response was fitted in a second-order polynomial equation:

$$Y = 286 + 33.81A + 96.66B + 17.85C\text{-}4.21D + 22.63AB + 48.36AC\text{-}16.37AD + 22.62BC + 38.33BD + 21.55CD\text{-}\ 35.78A^2\ \text{-}90.68B^2\ \text{–}0.388C^2\ \text{–}10.84D^2$$

Where A is the fermentation time, B is the moisture content, C is the size of inoculum and D is the particle size of CASP. This equation was used to correlate the impact of experimental variables with enzyme production. Model coefficients were estimated by multiple linear regressions and those with $P < 0.05$ were considered significant. It was observed that the linear effects of A, B, C; quadratic effects of A, B, D and interaction effects of AB, AC, AD, BC, BD and CD were significant for alkaline protease production and D, C^2 were found insignificant ($P > 0.05$). The highest protease activity in the experimental design was found to be 349.85 U/ml at 50% moisture, fermentation time of 144 h, inoculum level of 1×10^8 spores/ml and 1 mm particle size of CASP. These results displayed that the predicted and experimental values did not show significant difference ($P > 0.05$) suggesting the suitability of model for maximizing alkaline protease production.

The model F-value of 250.32 implied that model is significant ($P < 0.0001$). The "Lack of Fit F-value" of 1.83 implies the Lack of Fit is not significant relative to the pure error which is the desired attribute and signifies that data fits the model. The goodness of fit of the model was checked by determination coefficient (R^2). In this study, R^2 value for the model was found to be 0.996 while the adjusted and predicted R^2 values were calculated as 0.992 and 0.980. This indicated that only 0.04% of total variations cannot be explained by this model. Thus, the present R^2 value reflected a good fit between observed and predicted responses and implied that the model is reliable for predicting alkaline protease production.

3.2.1. Response surface plot

The regression equation presented as 3-D response surface plots depicting the interaction among the variables (Fig. 2). Here, each response surface plot represented the effect of two independent variables, holding the other variables at zero levels.

A concurrent relationship was seen when effect of fermentation time and moisture content was plotted against the enzyme activity, hence increase in moisture content with increase in fermentation time favours response up to the optimum level (Fig. 2a). It was seen that protease production was enhanced by increasing the moisture content of the medium up to 63% after which a decline in enzyme production was observed. These results are consistent with earlier reports. Germano et al. (2003) reported requirement of 55% moisture for protease production using *Penicillium* sp. in SSF. Similarly, de Castro et al. (2015a) also used 50% moisture for protease production using *Aspergillus niger*. Microbial growth and enzyme production in SSF is governed by the initial moisture content of the medium. Low moisture reduces water activity, diminishing growth of microorganism due to lack of availability of nutrients. Whereas, a comparatively higher moisture level impairs oxygen transfer and reduces porosity as the substrate particles tend to stick together (Uyar and Baysal, 2004). The response plot also showed that increasing the fermentation time till 123 h favoured optimum protease production from *Neocosmospora* sp. N1. Fermentation time is a prime factor defining an industrial process. Less fermentation period leads to high productivity and lowers the risk of contamination (Mazutti et al., 2007). However, Novelli et al. (2016) suggested that protease activity is associated with different fermentation times for different strains. Ali and Vidhale (2013) observed maximum protease production in *Fusarium oxysporum* in 72 h while Shivakumar (2012) reported 120 h fermentation time for optimum protease production from *Aspergillus* sp. Optimum fermentation time of 72 h was also reported for production of protease from *Penicillium* sp. by Agrawal et al. (2004).

The response surface plots determining optimum level of inoculum size suggested that increasing the inoculum level enhanced enzyme production and inoculum size of 1×10^8 spores/ml was found to be optimum for protease production (Fig. 2b, d, f). A positive correlation was seen when inoculum level interacted with moisture content

Table 5
Analysis of variance for the experimental results of Box-Behnken Design.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.108E+05	14	15056.55	250.32	<0.0001	significant
A-Fermentation Time	13719.42	1	13719.42	228.09	<0.0001	
B-Moisture Content	1.121E+05	1	1.121E+05	1863.90	<0.0001	
C-Inoculum Size	3822.40	1	3822.40	63.55	<0.0001	
D-Particle Size (CASP)	213.19	1	213.19	3.54	0.0807	
AB	2047.56	1	2047.56	34.04	<0.0001	
AC	9355.73	1	9355.73	155.54	<0.0001	
AD	1072.56	1	1072.56	17.83	0.0009	
BC	2047.56	1	2047.56	34.04	<0.0001	
BD	5875.22	1	5875.22	97.68	<0.0001	
CD	1858.47	1	1858.47	30.90	<0.0001	
A^2	8303.13	1	8303.13	138.04	<0.0001	
B^2	53342.49	1	53342.49	886.82	<0.0001	
C^2	0.9765	1	0.9765	0.0162	0.9004	
D^2	762.80	1	762.80	12.68	0.0031	
Residual	842.10	14	60.15			
Lack of Fit	690.92	10	69.09	1.83	0.2945	not significant
Pure Error	151.18	4	37.80			
Cor Total	2.116E+05	28				

*$R^2 = 0.996$, R^2 (pred) = 0.980, R^2 (adj) = 0.992.

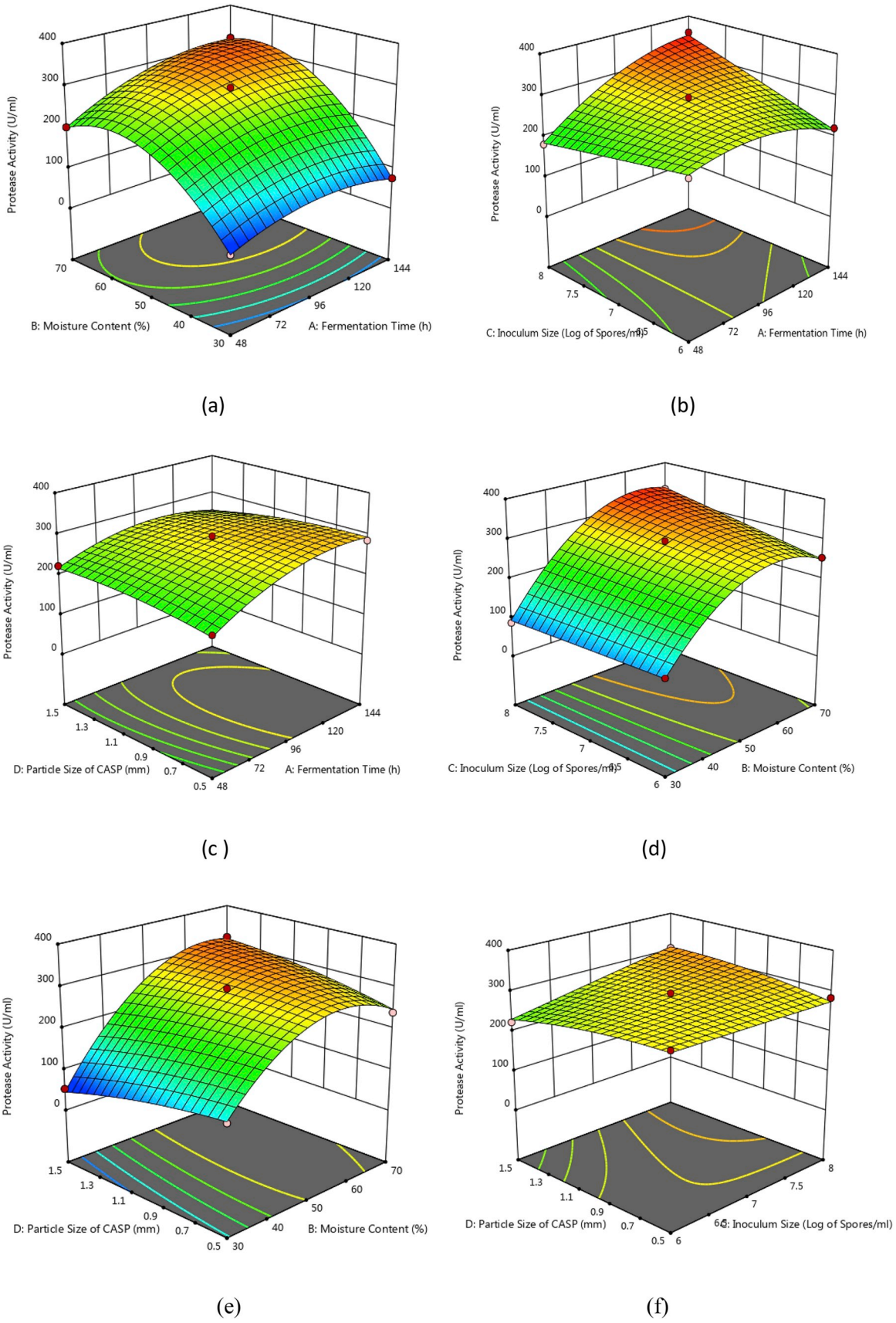


Fig. 2. Response surface plots showing interaction effects of (a) fermentation time and moisture content (b) fermentation time and inoculum size (c) fermentation time and particle size (d) moisture content and inoculum size (e) moisture content and particle size (f) inoculum size and particle size.

(Fig. 2d). Earlier reports demonstrated that size of inoculum has a profound effect on growth of microorganism and enzyme production since a lower inoculum size may result in insufficient biomass for optimum product formation (Pandey et al., 2000) while a higher inoculum level increases competition between cells resulting in reduced mass transfer capacity thus decreasing enzyme production (Limkar et al., 2019; Reddy et al., 2008).

Fig. 2c and f represents the horizontal surface plots obtained between particle size of CASP interacting with fermentation time and inoculum level, respectively. The graph reflects minimal interaction of particle size with these variables while a slight inclined curve was observed between the particle size of CASP and the moisture content (Fig. 2e) suggesting significant interaction amongst them. Thus, increasing the particle size of CASP decreased protease production and optimum level was predicted at 1.4 mm. The particle size of substrates used in SSF largely affects enzyme production. Smaller substrate particles provide a larger surface area facilitating growth of microbes; however, too small particles may result in substrate agglomeration resulting in retarded growth. Alternatively, larger particles limit microbial growth due to reduction in surface area, poor aeration and mass transfer (Pandey et al., 2000). Ali and Vidhale (2013) used rice bran with 0.85 mm mesh size while Prakasham et al. (2006) used 1.4–1.0 mm particle size of green gram husk for protease production.

3.2.2. Validation of the model

The validation of the experimental model was conducted by performing fermentation under the predicted conditions. The optimised values of four variables taken under consideration are fermentation time 123 h, moisture content 63%, inoculum size 1×10^8 spores/ml and particle size 1.4 mm. The predicted response was calculated to be 338.9 U/ml. The growth kinetics and protease production of the culture were studied using this optimised medium (Fig. 3). Protease production increased with the rate of growth and maximum production was observed towards the onset of stationary phase after which it remained constant throughout the study. Microbial growth associated with biosynthesis of protease has long been debated. Soares et al. (2005) proposed that protease production is growth associated and maximum enzyme production is observed at mid exponential phase. On the contrary, Gupta et al. (2002) suggested that proteases are known to be associated with the onset of stationary phase, which is marked by the transition from vegetative growth to sporulation stage in spore-formers. This study supported the fact that extracellular protease production is a manifestation of nutrient limitation at the onset of stationary phase. Maximum protease activity of 364.4 U/ml was achieved using the standardised conditions. There was a high degree of similarity between predicted and observed experimental value verifying that the model prediction was in good agreement with the experimental data, validating the model design. Statistical optimization of culture parameters resulted in 3.12 fold increase in protease production over unoptimised conditions.

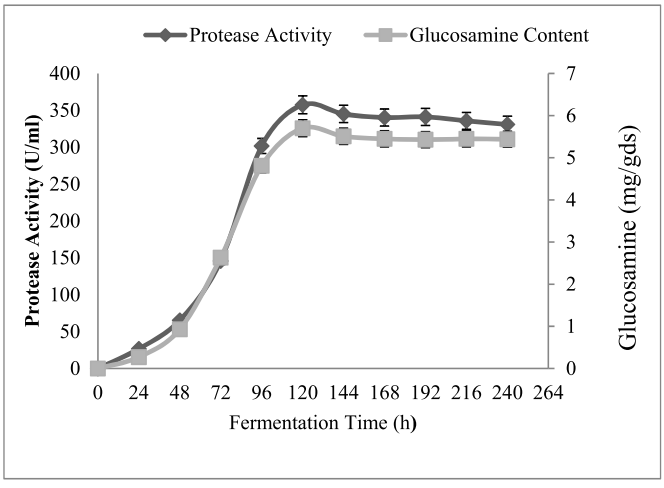


Fig. 3. Growth kinetics and protease production of *Neocosmospora* sp. N1 in optimised medium.

Numerous studies portray the use of response surface methodology for optimization of medium components and physiochemical parameters as it is more effective than the conventional methods of optimization, saves time and cost invested in attaining optimised conditions. Mishra (2016) used Box-Behnken Design to optimize medium and fermentation conditions for *Brevibacillus brevis* and attained 1.5 fold enhancement in alkaline protease production. Wahab and Ahmed (2018) achieved a 3.6 fold hike in enzyme production using Central Composite Design (CCD) for optimization of protease production by *Aspergillus niger*. Patel et al. (2019) optimised medium for *Bacillus subtilis* using CCD and obtained up to 4 fold increase in protease production over basal medium. Every organism has a unique requirement for these parameters therefore each one has to be considered individually in order to maximize enzyme production. (Suberu et al., 2019; Mechri et al., 2017). To the best of authors' knowledge, this is the first ever contribution towards use of CASP as an inducer for production of protease by using *Neocosmospora* sp. N1 and its statistical optimization.

3.3. Partial purification and characterization of protease

The protease enzyme was purified from crude extract by using 0–90% ammonium sulphate fractionation and desalted over Sephadex G-25 chromatography column. It was concentrated by reverse dialysis against solid sucrose having protease activity of 2030.6 U/ml and specific activity of 59.09 U/mg. The partially purified enzyme was optimally active over pH 8 to12 and temperature 60 °C (Fig. 4). Jain et al. (2012) reported an alkaline protease from a *Bacillus* sp. having optimum temperature of 60 °C and pH 10. Similar results were reported by Thakrar and Singh (2019) for a protease produced from *Nocardiopsis* sp. The partially purified alkaline protease obtained in this study was used further in application studies.

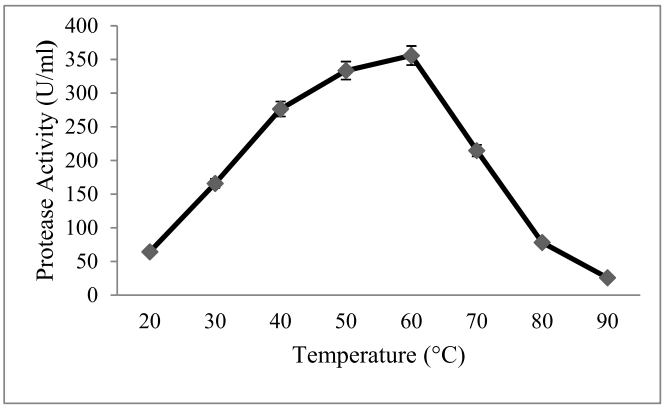
3.4. Wash performance studies

3.4.1. Compatibility with laundry detergents

The suitability of an enzyme as a detergent additive depends on its compatibility with various laundry detergents. The data presented in Fig. 5A showed that the protease used in this study was extremely stable in the presence of detergents like Ariel, Tide, Chameli and Badshah retaining more than 90% activity even after 3 h of incubation. The activity of protease slightly diminished when incubated with Surf Excel and Wheel, where enzyme retained 80% activity after incubation under similar conditions. Hammami et al. (2018) reported a protease from *Bacillus mojavensis* which was stable in detergents like Ariel, Tex'tile and Carrefour, retaining 100% activity when incubated for 1 h at 40 °C. Benmrad et al. (2018) isolated a protease from *Penicillium* sp. which retained 100% activity in Tide, Ariel and OMO, while Germano et al. (2003) reported only 60% activity of protease from *Penicillium* in OMO detergent when incubated for 1 h at 28 °C. A protease from *Aspergillus niger* was stable for 1 h at 40 °C in Ariel, Tide and Lange detergents (Wahab and Ahmed, 2018). The performance of a protease in detergent depends on number of factors, including the detergent compounds, thus the stability of this protease also varied with each laundry detergent. The above result suggested that protease produced from *Neocosmospora* sp. N1 can find utility as a detergent additive.

3.4.2. Blood stain removal

Stain removal ability of the protease was further evaluated using blood stained cloth pieces. The results of cloth pieces treated variedly are displayed in Fig. 5B. A limited washing performance was observed with detergent treatment as compared to enzyme treatment alone,



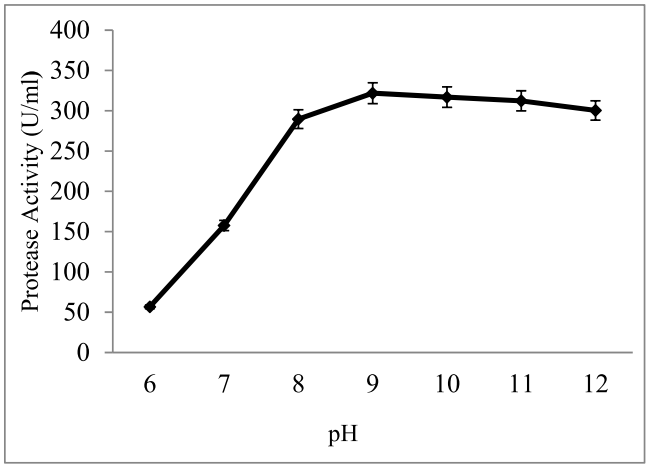


Fig. 4. Effect of (A) temperature and (B) pH on enzyme activity of partially purified protease.

while, the stain was completely removed when the cloth piece was treated with the mixture of detergent and enzyme. Supplementation of this protease in detergent improves its washing performance sustaining its utility in future industrial applications as a bioadditive in detergent formulations. The washing performance of alkaline proteases from *Bacillus* sp. is well documented (Rao et al., 2009). However, only a few alkaline proteases produced from fungal species are reported for their stain removal ability. Benmrad et al. (2018) reported a protease from *Penicillium chrysogenium* which could remove blood stain in 1 h when incubated along with detergent at 40 °C. An alkaline protease from *Botrytis cinerea* was able to remove blood stain when incubated at 60 °C for 15 min along with detergent (Abidi et al., 2008).

3.4.3. *Fourier-transform infrared spectroscopy analysis*

FTIR was used to analyse the effect of detergent and enzyme treatment on cotton fibre after stain removal. The data comparing different spectrograms is depicted in Fig. 5C (the red spectrum defines cotton treated with detergent, blue spectrum shows cotton treated with protease and black spectrum represents the original fabric). A number of peaks, characteristic of cotton cloth were assigned in the spectrum. Intense peaks of 3300.72 cm^{-1} and 1711.62 cm^{-1} indicated the presence of hydroxyl group (OH) and carbonyl group (CO). Absorption around 1630–1700 cm^{-1} showed protonation of the ionized carboxylate group (COO) and the peaks between 1400 and 1420 cm^{-1} are associated with HCH and OCH stretching vibration. Another peak at 1338.53 cm^{-1} indicated the presence of halogenated derivatives (CF) (Fan et al., 2012). The overall spectrum of control cloth and enzyme treated cloth did not show much difference; however, there was a significant increase

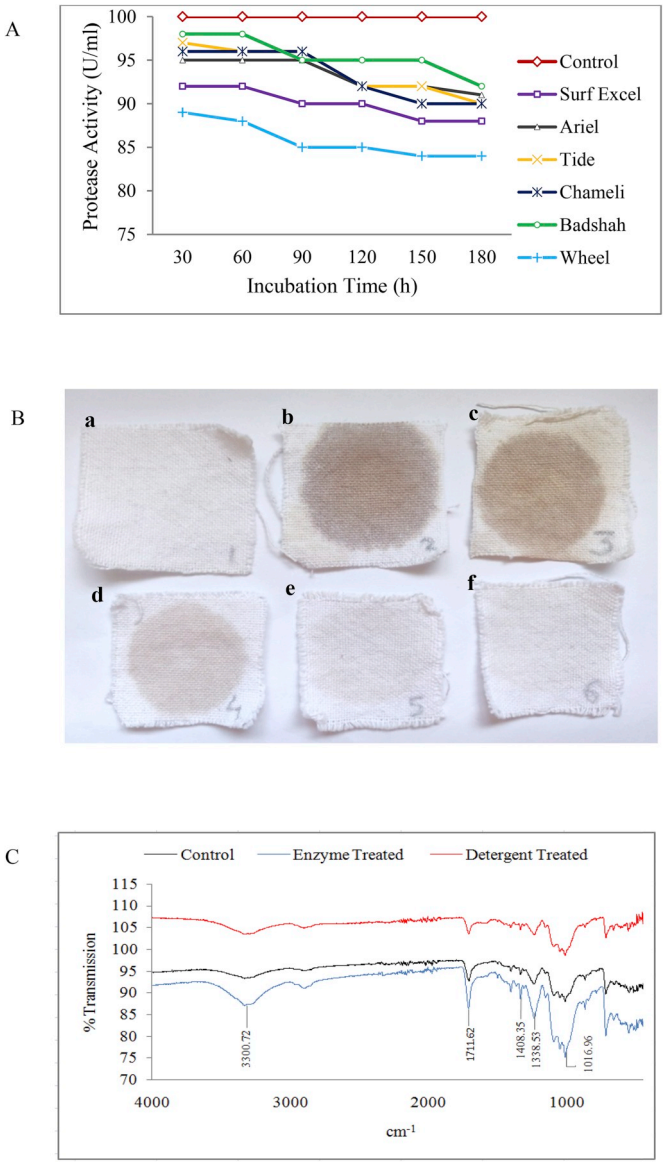


Fig. 5. (A) Relative activity of alkaline protease in presence of various laundry detergents (7 mg/ml) at different time intervals. Enzyme activity in absence of detergent was considered as control. (B) Wash performance analysis of protease. (a) original cotton cloth piece (b) blood stained cloth; stained cloth pieces washed with (c) water, (d) commercial detergent (7 mg/ml), (e) protease (500 U/ml) and (f) mixture of detergent (7 mg/ml) + protease (500 U/ml). (C) FTIR spectrogram of original cloth (black), stained cloth washed with detergent (red) and stained cloth washed with enzyme (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in % transmission of detergent treated cloth spectrum when compared to the control spectrum. Laundry detergents are composed of harsh chemicals which interact with cloth fibres during washing and eventually weaken them. This is well justified in the spectra obtained from our FTIR analysis. The increase in peak intensity of detergent treated cloth spectrum accounts for lesser number of bonds. Therefore, the fabric can lose its strength by repeated washing with detergent. The outstanding capability of our protease to remove stain without making structural changes on the fabric amplifies its efficacy in detergent industries. In a similar study, stained cloth washed with protease produced from *Penicillium chrysogenium* did not impart structural change on the fabric (Benmrad et al., 2018).

3.5. Dehairing studies

3.5.1. Removal of hairs from goat skin

Leather industries have started adapting the use of enzymatic dehairing of animal hides for reducing environment pollution. In this study, goat skin pieces were dehaired using protease produced from the fungal isolate, *Neocosmospora* sp. N1 and compared with the chemical treatment (lime and sulphide) used as conventional method in leather industries (Fig. 6A). Hairs were easily removed after 24 h of incubation in both enzymatic and chemically treated skin as compared to control. Upon visual assessment, the enzyme treated skin had cleaner pores, smooth grain structure, white and softer appearance in contrast to the chemical treated skin which turned black, harder with residual hair visible in some of the hair pores. Earlier scientific reports have addressed the applicability of *Bacillus* derived alkaline protease for dehairing of animal skin (Hammami et al., 2018; Briki et al., 2016; Tiwary and Gupta, 2010). The protease produced from *Neocosmospora* sp. N1 was comparable to *Bacillus* derived proteases and can find utility in leather industries.

3.5.2. Histological staining and scanning electron microscopy

Histological sections of untreated goat skin and dehaired pelts from enzymatic and chemical treatments are shown in Fig. 6B after staining with hematoxylin-eosin stain. The control skin showed the presence of intact epidermis and collagen fibres. The absence of epidermis and empty follicles appearance was evident in the treated skin. The skin piece treated with enzyme had little effect on collagen fibres indicating that the enzyme was devoid of collagenase activity while the collagen structure in papillary dermis was completely damaged as a result of chemical treatment on skin. The result was further supported by SEM images revealing the structural change in upper dermal layer of treated skin (Fig. 6C). Saleem et al. (2012) described the use of an alkaline protease from *Bacillus cereus* in dehairing of animal hides with absence of collagenase activity. George et al. (2014) used alkaline protease from *Vibrio metschnikovii* for dehairing of goat skin without having collagenase activity. The inactivity of protease on skin collagen is one of the prerequisite for its application in dehairing as damage to the collagen fibres of the grain layer imparts unfavourable properties to finished leather (Haddar et al., 2011).

4. Conclusion

Microbial proteases dominate the enzyme industry, contributing two third of the total enzyme sales. The rising demand of proteases necessitates the search for novel proteases and allows researchers to develop effective technologies which can circumvent the major cost associated with enzyme production. This study focuses on optimising the production of alkaline protease using a novel fungus, *Neocosmospora* sp. N1. The enzyme was optimally active at 60 °C and a broad range of pH 8 to 12. The use of readily available agro-waste material WB in the fermentation process could possibly reduce major cost associated with the enzyme production in industries. The production of protease was enhanced up to 1.88 fold using another agro-waste CASP as a novel inducer in the medium. Further, the process parameters were statistically optimised to attain maximum alkaline protease production of 364.4 U/ml. The cost effective production with appreciable yield of

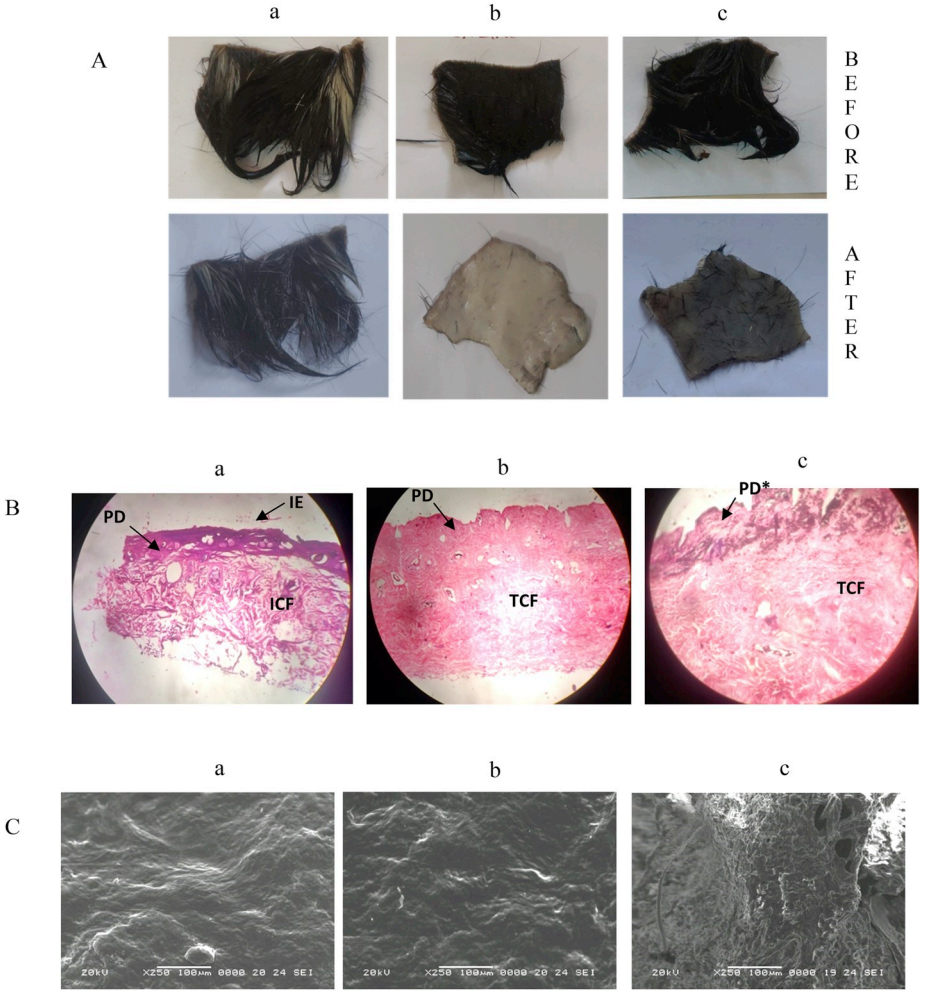


Fig. 6. (A) Goat skin pieces treated with (a) buffer (b) protease (500 U/ml) in buffer solution and (c) 6% lime and 3.5% sulphide solution, incubated for 24 h at 40 °C. (B) SEM image of (a) control skin, (b) enzyme treated skin and (c) chemical treated skin at 250x magnification. (C) Histological staining of tissue section obtained from (a) control skin (b) enzyme treated skin and (c) chemical treated skin. Micrographs were taken at 10x magnification using a light microscope. IE, PD, PD*, ICF and TCF represents intact epidermis, papillary dermis, damaged papillary dermis, intact collagen fibres and treated collagen fibres, respectively.

protease from *Neocosmospora* sp. offers a great deal of advantage to industries, therefore, further work on large scale production can be carried out in future. The efficiency of crude protease was evaluated for industrial applicability. The enzyme showed excellent compatibility with commercial detergents and was potent in removing blood stain from cotton fabric. The protease also exhibited outstanding dehairing property and proved better than the chemical treatment used in leather industries. Owing to the thermostability and alkaline nature, this protease can serve as a promising tool in detergent, leather, food and textile industries. However, the enzyme has restricted use at low temperature and acidic environment.

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Declaration of interest

None.

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