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Enhanced production of alkaline protease by *Aspergillus niger* DEF 1 isolated from dairy form effluent and determination of its fibrinolytic ability

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Proteases constitute most important enzymes owing to their wide variety of functions and have immense applications in various industries *viz*., medical, pharmaceutical, biotechnology, leather, detergent, and food industries. Despite of their wide spread occurrence in various sources, microorganisms present remarkable potential for proteolytic enzymes production due to their extensive biochemical diversity and susceptibility to genetic manipulation. The present study was aimed at isolating alkaline protease producing fungal members from dairy form effluents, designing the process variables for maximizing the protease production and determining the fibrinolytic potential of the partially purified alkaline protease. To achieve the specified objectives, the dairy form effluent was processed for the isolation of proteolytic fungi using suitable microbiological medium. All the fungal isolates were screened for their protease producing ability and the isolate showing highest alkaline protease production was selected for further studies. Optimization of different fermentative variables like carbon, nitrogen sources, pH, temperature and incubation period were carried out to enhance enzyme production. Ammonium sulphate fractionation was employed to partially purify the enzyme following which its fibrinolytic potential was determined. Based on morphological and microscopic studies, the selected fungal isolate was identified as *Aspergillus niger*. Optimization studies using OVAT (one variable at a time) method revealed an enhanced protease production in the presence of fructose as additional carbon source and ammonium sulphate as nitrogen source. The optimum incubation period, temperature and pH for enzyme production by the selected fungal isolate was found to be 92 h, 50°C and 10, respectively. The partially purified alkaline protease was efficient in the removal of blood stains emphasizing its fibrinolytic ability. An alkaline protease producing Fungal sp. was screened and isolated from dairy form effluent and it was found to be efficient in the removal of blood stains proving its fibrinolytic potential. Enzymes produced from microorganisms that can survive under extremes of pH could be particularly useful for commercial applications under high alkaline conditions.

Key words: Alkaline protease, dairy form effluent, optimization, *Aspergillus niger*, fibrinolytic potential.

INTRODUCTION

Proteases or proteinases are enzymes which carryout proteolysis by the hydrolysis of their peptide bonds (Anshu and Khare, 2007; Kalpana Devi et al., 2008). Among the industrial enzymes, proteases are one of the

most important groups contributing to the largest sales after carbohydrases and they account for a quarter of the total global enzyme production (Kalaiarasi and Sunitha, 2009). They are ubiquitous and widely distributed in plants, animals and microbes with microbes as major sources among all (Amrita Raj et al., 2012). Fungi harbor a variety of proteolytic enzymes and are important sources compared to bacteria because of their ability to grow at extremes of temperature, pH and as well use a wide variety of substrates as nutrients. Their importance is mainly due to their wide spread applications in various industries *viz*., detergent industry, leather processing, food industries, textile industry, pharmaceutical etc. (Deng et al., 2010; Jellouli et al., 2009). Considering the importance of proteases it is necessary to screen and isolate novel protease producing microbes from different sources. It is ideal that proteases isolated from microbes should maintain higher activities over a broad range of temperature, pH etc. to be used in various industrial applications (Johnvesly and Naik, 2001). Thermo stable enzymes, in addition to their temperature resistance have additional advantages including: Increasing the diffusion and mass transfer rate as the viscosity decreases at high temperatures, enhancement of the reaction rate constant, and increasing the solubility of the hydrophobic substrates. Besides these, high temperatures also lower the risk of contamination by pathogenic microbes (Al-Qodah et al., 2013). Enzymes from microorganisms that can survive under extreme pH may be useful for commercial applications under high alkaline reaction conditions, e.g., in the production of detergents (Denizci et al., 2004). Based on their optimal pH, proteases are categorized into neutral, acidic and alkaline proteases and among them alkaline proteases have major application in the detergent industry as the pH of the detergents usually lies in the range of 9 to 12. Thus, search for new proteases from different sources with novel properties is desirable in order to produce proteases of high performance (Al-Qodah et al., 2013).

In general the effluents of meat, dairy form and poultry industries serve as prime sources of proteolytic microbes owing to their protein rich content (Vishwanatha et al., 2010). In the present study, proteolytic fungi were screened and isolated from dairy form effluent; process variables were optimized for maximizing alkaline protease production and the ability of selected isolate was tested for its fibrinolytic activity.

MATERIALS AND METHODS

Sample collection and isolation of proteolytic fungi

Dairy form effluent was aseptically collected from the Creamline Dairy Products Ltd., Epuru, using sterile spatula and the effluent was carefully packed in a zip lock cover that was pre-sterilized. The collected sample was processed within an hour or stored in the refrigerator if not immediately used. Alkaline protease producing fungal strains were isolated by subjecting the effluent to 10 fold serial dilution and by inoculating the serially diluted sample on modified seed medium (Reese et al., 1950) of pH 9.0. Table 1 shows the composition of modified seed medium. The plates were then incubated at 40°C for a period of 5 days. The alkaline protease producing fungal isolates were identified based on zone of casein hydrolysis around the colonies.

Identification of the fungal isolates

The alkaline protease producing fungi were identified based on morphological characteristics and microscopic observation of fungal spores using lactophenol cotton blue staining. For morphological characterization, the fungal isolates were cultivated on czepakdox agar medium. The shape, size, arrangement and development of conidiophores, phialides and conidiospores were studied using the taxonomic tools of Hoog et al. (2000).

Protease assay

The proteolytic activity of the isolated fungal species was estimated by the method of Udandi Boominadhan et al. (2009). The enzyme activity was estimated in a reaction mixture containing 2.0 ml of 0.5% casein solution in 0.1M Carbonate –Bicarbonate buffer (pH 9.5) and 1 ml crude enzyme extract in a final volume of 3.0 ml. The reaction mixture was incubated at 40°C for a period of 5 min followed by termination of the reaction by the addition of 3 ml of ice-cold 10% TCA (Tri Chloro Acetic acid). After 1 h incubation at room temperature, the precipitate so formed was collected by filtering the contents using Whattman no. 1 filter paper. The tyrosine released by the action of crude enzyme extract on protein was estimated by adding 5 ml of 0.4 M Sodium carbonate and 0.5 ml of Folin phenol reagent to 1 ml of filtrate. This also aid in color development. The mixture was then vortexed immediately and incubated for 20 min at room temperature. The tyrosine released was estimated by measuring OD at 660 nm against the enzyme blank using UV-VIS spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µg of tyrosine per mL per min under standard assay conditions.

Optimization of process parameters by one variable at a time (OVAT) method

The alkaline protease production by the fungal isolate (DEF 1) yielding highest protease was further optimized using OVAT method. The production medium (Nehra et al., 2002) consists of (g L^{-1}) Glucose (10), Casein (5), Yeast extract (5), Ampicillin (0.05), K_2HPO_4 (1), $MgSO_4.7H_2O$ (0.2), Na_2CO_3 (10) and pH (8.5). For optimization, the production medium was supplemented with different additional carbon sources 1% (w/v) (glucose, fructose, sucrose and maltose) and 1% (w/v) nitrogen sources (peptone, yeast extract, beef extract, ammonium sulphate, ammonium chloride and sodium nitrate). The effect of various physical parameters such as pH (5, 6, 7, 8, 9, 10, 11 and 12), temperature (25, 30, 35, 40, 45 and 50°C) and incubation time (48, 72, 96 and

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Table 1. Composition of modified seed medium.

Media component	Quantity (g/L)
Glucose	2.5
Casein	5
Ampicillin	0.05
pH	9

Fungal members were screened by inoculating dairy form effluent on modified seed medium and incubating the plates at 40°C for a period of 5 days.

Figure 1. Creamline dairy products Ltd. Epuru, Hanuman Junction, Krishna district.

120 h) were also optimized by OVAT method. Submerged Fermentation (SmF) studies were carried out in 250 mL Erlenmeyer flasks containing 45 mL production medium inoculated with 3% inoculums and incubated for 4 days in an orbital shaker (150 rpm) at 40°C. Fungal biomass was separated by centrifugation at 10,000 rpm for 10 min and the filtrate was considered as a crude enzymatic extract and stored at -20°C for further use.

Partial purification of protease

The crude protease extract obtained by culturing *A. niger* DEF 1 in the optimized protease production medium was subjected to ammonium sulphate fractionation (0-20, 20-40, 40-60 and 60-80%). The precipitates so obtained by centrifugation were dissolved in 0.1 M phosphate buffer (pH 7) and dialyzed against same buffer for overnight at 4°C. The samples were then tested for protease activity using protease assay.

Determination of fibrinolytic activity of partially purified protease

Fibrynolytic potential of partially purified protease was determined by incubating 3 pieces of cotton fabric impregnated with blood. The dried blood stains were fixed with 2% (v/v) formaldehyde for 30 min and the excess formaldehyde is removed by rinsing with water (Adinarayana, 2003). The cotton fabrics were then allowed to dry. After drying, the cotton fabrics were incubated separately with 2 ml of partially purified protease along with detergent, 2 ml of distilled water with detergent and 2 ml of distilled water at 40°C for 1 h. The cotton fabrics were then rinsed with water and checked for the removal of blood stains following drying.

Statistical analysis

Effect of each variable was studied in triplicates and the results were graphically presented as the mean ± standard deviation.

RESULTS

Isolation and screening of proteolytic fungi

A total of 7 different alkaline protease producing fungal sp. were screened and isolated from dairy form effluents collected from Creamline Dairy Products Ltd. Epuru. Figure 1 shows the Creamline Dairy Products Ltd situated at Epuru, near Hanuman Junction, Krishna District. Figure 2 shows the huge heap of left over milk packets dumped in the dairy form premises. Table 2 shows the fungal sp. isolated from the dairy form effluent. Figure 3 shows the isolated fungal sp. (DEF 1 to DEF 7). The proteolytic potential of the fungal isolates was evident by the formation of clear zones around the colonies on skimmed milk agar medium (Figure 4). Of the isolated fungal sp. DEF 1 exhibited highest enzyme production (94 U/mL) and highest zone of proteolysis (20 mm) compared to other isolates (Tables 3 and 4). So it was selected for further optimization studies.

Identification of the selected fungal isolate

The highest protease producing fungal isolate was identified as *A. niger* based on the morphological characters and microscopic observation of spores using lactophenol cotton blue. The morphological and microscopic characteristics of the selected isolate were given in Table 5.

Optimization of process parameters using OVAT method

Effect of incubation period

As the growth phase of the fungus lasts for about 7 to 10 days and the log phase lies in between 3 and 5 days, it is our interest to determine the time of incubation at which lipase production is maximum by *A. niger* DEF 1. So the protease production as factor of incubation time was studied using shake flasks up to 144 h. From the data represented in Figure 5a, it was evident that maximum enzyme yield was obtained after 96 h of incubation period.

Figure 2. A huge heap of milk packets thrown in the dairy form premises.

Table 2. List of fungal members isolated from dairy form effluent.

S/N	Fungal isolate
DEF 1	*Aspergillus* sp.
DEF 2	*Rhizopus* sp.
DEF 3	*Mucor* sp.
DEF 4	*Penicillium* sp.
DEF 5	*Aspergillus* sp.
DEF 6	*Aspergillus* sp.
DEF 7	*Fusarium* sp.

*DEF = Dairy form effluent fungi. 7 different fungal isolates were screened and isolated from dairy form effluent inoculated on modified seed medium.

Effect of incubation temperature

Temperature of the medium plays an important role on metabolic activities of living organisms, in the present case on protease production. As the low temperature shuts or slows down the metabolic activity and high temperature inhibits or deactivates the enzymes or hormones, we studied the effect of temperature on enzyme production by incubating the fungus at different temperatures from 25 to 70°C and from the results obtained it was found that the enzyme production was maximum at 50°C (Figure 5b).

Effect of the medium pH

pH of the medium is an important parameter that affects the growth and metabolism of the organism. Most of the organisms prefer pH from range 5 to 8 for their optimal growth. However, few prefer acidic pH and few others prefer basic pH based on their secretory products. Hence in the present study, the effect of pH on lipase production was studied by culturing the fungus in the protease production medium for 96 h with a pH range 5 to 11 and from the results obtained it was found that optimum pH for protease production was pH 10 (Figure 5c).

Effect of additional carbon source

Carbohydrates like glucose, fructose and sucrose are the most preferred carbon sources to any organisms as they can be readily used up. To check whether carbon source in the form of carbohydrates affect the lipase production, various sugars *viz*., glucose, fructose, maltose and sucrose at a concentration of 1% were added as additional carbon sources to protease production medium. The data obtained (Figure 5d) indicates that fructose enhanced the enzyme production compared to other carbon sources.

Effect of nitrogen sources

The growth of the microbes and enzyme production requires nitrogen sources. Most of the microorganisms require fixed nitrogen sources to synthesize proteins, nucleic acids and other cellular components. The nitrogen sources may be provided as pre-digested polypeptides, as bulk proteins or as nitrate or ammonium salts depending upon the enzyme capabilities of the microorganisms. Hence in the present study, the effect of nitrogen sources on protease production by *A. niger* DEF 1 was studied by incubating the organism in protease production medium with various organic nitrogen sources (1%) *viz*., peptone, yeast extract, beef extract etc. and

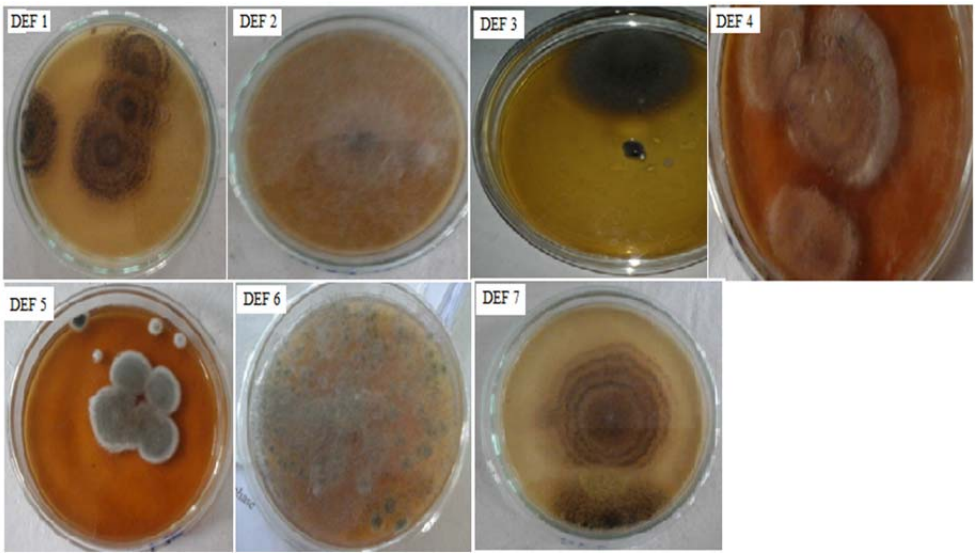

Figure 3. Fungal sp. isolated from dairy form effluent.

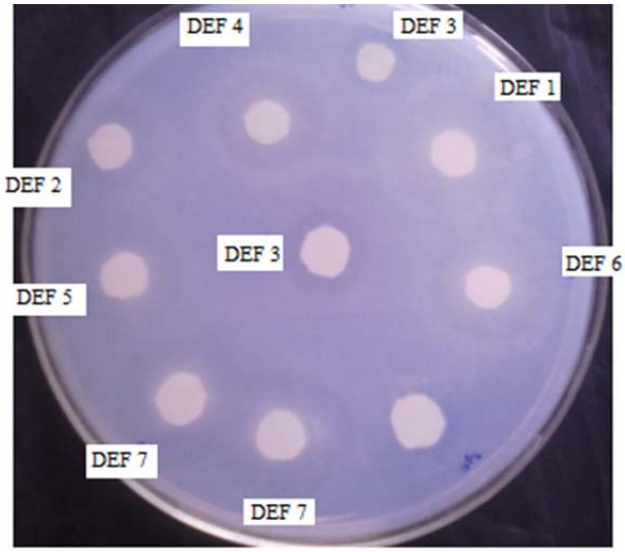

Figure 4. Pertriplate showing zone of proteolysis by the fungal species isolated from dairy form effluent.

with various inorganic nitrogen sources (1%) *viz.,* ammonium sulphate, ammonium chloride and sodium nitrate. From the data obtained (Figure 5e), it was evident that the best nitrogen source for protease production by *A. niger* DEF 1 was found to be Ammonium sulphate.

Partial purification of protease

The highest protease activity was found with 40 to 60% precipitate and hence it was used for further studies.

Fibrinolytic potential of the protease

The fibrinolytic potential of partially purified protease was found in terms of degree of blood removal from the cotton fabric and from the results obtained, blood removal was found in the order of partially purified protease with detergent > distilled water along with detergent > distilled water (Figure 6).

DISCUSSION

Proteases, having large number of commercial applications account for more than 60% of the total enzyme market. They are the oldest enzymes known to mankind and catalyze the hydrolysis of proteins (Raju et al., 1994). They are widely distributed in the nature and are very much essential for cell growth and differentiation (Vadlamani and Parcha, 2011). Despite of their wide spread occurrence in a variety of sources, microbes have proven to be common sources due to their biochemical and physiological properties, ease of genetic manipulation and facile culture conditions. Many researchers are continuously engaged in screening for potential protease producers as these enzymes constitute 2/3rd of the total enzymes used by various industries and this demand is supposed to increase as the years pass by. In recent years also there were several reports on screening and isolation of protease producers (Chandrasekaran et al., 2015; Sharma et al., 2015; Sonia sethi et al., 2015; Rupali et al., 2015; Tiwari et al., 2015) indicating the importance of these industrial enzymes. Hence the present study was undertaken to isolate potential alkaline protease producing fungi from dairy form effluents as these effluents provide alkaline environment for the

Table 3. Fungal isolates and their corresponding protease activity in units/mL.

S/N	Fungal Isolates	Protease activity (Units/mL)
DEF 1	*Aspergillus* sp.	94
DEF 2	*Rhizopus* sp.	20
DEF 3	*Mucor* sp.	15
DEF 4	*Penicillium* sp.	36
DEF 5	*Aspergillus* sp.	27
DEF 6	*Aspergillus* sp.	82
DEF 7	*Fusarium* sp.	86

For the estimation of protease activity, the fungal spores were inoculated in 45 mL of production medium (pH 8.5) and the flasks were incubated at 40°C for a period of 72 h. The fungal biomass was then separated by centrifugation at 10,000 rpm for 10 min and the filtrate was considered as a crude enzymatic extract

Table 4. Fungal isolates and their corresponding protease activity in mm.

S/N	Fungal Isolate	Protease activity (mm)
DEF 1	*Aspergillus* sp.	20
DEF 2	*Rhizopus* sp.	7
DEF 3	*Mucor* sp.	5
DEF 4	*Penicillium* sp.	15
DEF 5	*Aspergillus* sp.	10
DEF 6	*Aspergillus* sp.	17
DEF 7	*Fusarium* sp.	19

The qualitative test for protease activity was done by placing the filter paper discs immersed in crude enzyme extract on skim milk agar medium following incubation of the plates at 40°C for a period of 24 h. The zone of casein hydrolysis around the disc was identified as protease activity and was measured in mm.

growth of alkaline protease producers.

In the present study, effluent was collected from Creamline Dairy Products Ltd. Epuru, Krishna District. 7 different alkaline protease producing fungal members were screened and isolated from the effluent samples using skim milk agar medium. The fungal isolate producing highest protease was found to be *A. niger* DEF 1. Alkaline protease production by *A. niger* was also reported by earlier workers (Kalpana Devi et al., 2008; Coral et al., 2003; Abidi et al., 2014) from different sources. For the first time, we are reporting an alkaline protease producing *A. niger* from dairy form effluent. The highest protease production was found in the presence of fructose as additional carbon source compared to glucose which could be due to catabolite repression by high glucose available in the production medium. Mukhtar and Ikram-Ul-Haq (2009) also reported the same observation with acid protease from *A. niger*. Higher protease yields were reported by several workers in the presence of a variety of carbon sources such as glucose, 1% wheat bran, 1% starch and corn steep liquor etc (Malathi and Chakraborty, 1991; Kalpana Devi et al., 2008; Vaishali and Jain, 2012; Palanivel et al., 2013).

Protease production is known to be influenced by the presence of nitrogen sources in the production medium and different organisms prefer different nitrogen sources for their growth and enzyme production (Singh et al., 2011). Earlier studies revealed significant protease production in the presence of organic nitrogen sources (Malathi and Chakraborty, 1991; Mukhtar and Ikram-Ul-Haq, 2009; Mostafa El-Sayed et al., 2012; Palanivel et al., 2013; Sonia sethi et al., 2015) and reduced growth and enzyme production in the presence of inorganic nitrogen sources such as ammonium compounds (Sehar and Hameed, 2011). In contrary to their findings, the present study reported higher protease production in presence of ammonium sulphate. Similar finding was also reported by Kalpana Devi et al. (2008). There were so many reports for higher protease production in the presence of inorganic nitrogen sources (Rajkumar et al.,

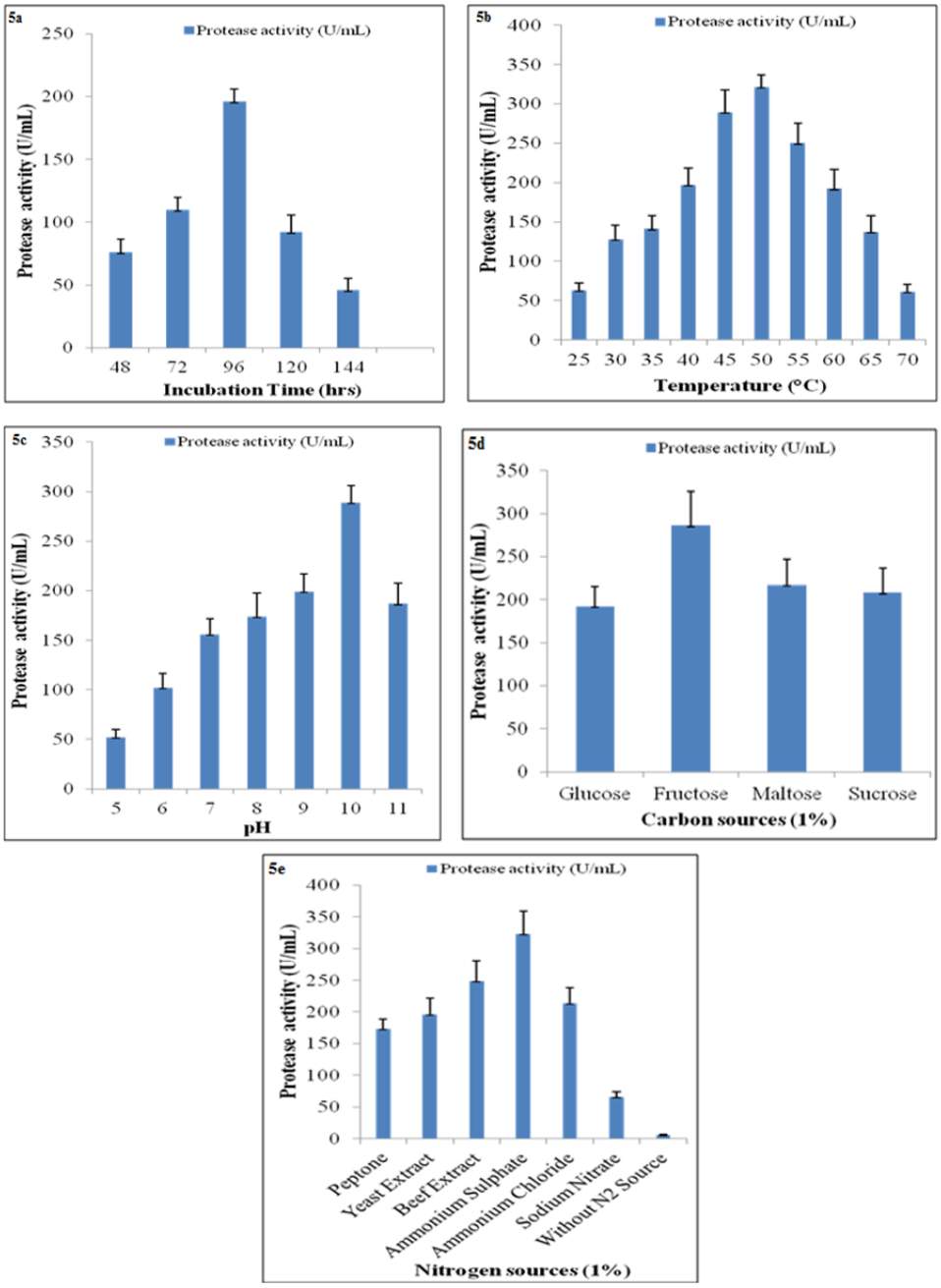

Figure 5. Optimization of process variables for enhanced alkaline protease production by *A. niger* DEF 1. (a) Effect of incubation period on protease production - Spore suspension was inoculated into production medium and incubated for different time periods viz., 48, 72, 96, 120 and 144 at 40°C followed by enzyme estimation. (b) Effect of incubation temperature on protease production - Spore suspension was inoculated into production medium and incubated at different temperatures from 25 to 70°C for 96 h at 150 rpm and enzyme activity was estimated. (c) Optimization of pH for protease production - Spore suspension was inoculated into production medium with varying pH ranging from 5 to 11 and incubated at 50°C for 96 h at 150 rpm and enzyme activity was estimated. (d) Effect of additional carbon sources on protease production - Spore suspension was inoculated into production medium of pH 10 with 1% of various sugars as additional carbon sources viz., glucose, fructose, maltose and sucrose etc. and incubated at 50°C for 96 h and enzyme activity was estimated. (5) Effect of nitrogen sources on protease production - Spore suspension was inoculated into production medium of pH 10 containing 1% fructose as additional carbon source with 1% each of various organic and inorganic nitrogen sources viz., Peptone, Yeast extract, Beef extract, ammonium sulphate, ammonium chloride and sodium nitrate etc. incubated at 50 °C for 96 h and enzyme activity was estimated. The production medium without nitrogen was used as control to show the importance of nitrogen for cell growth and enzyme production.

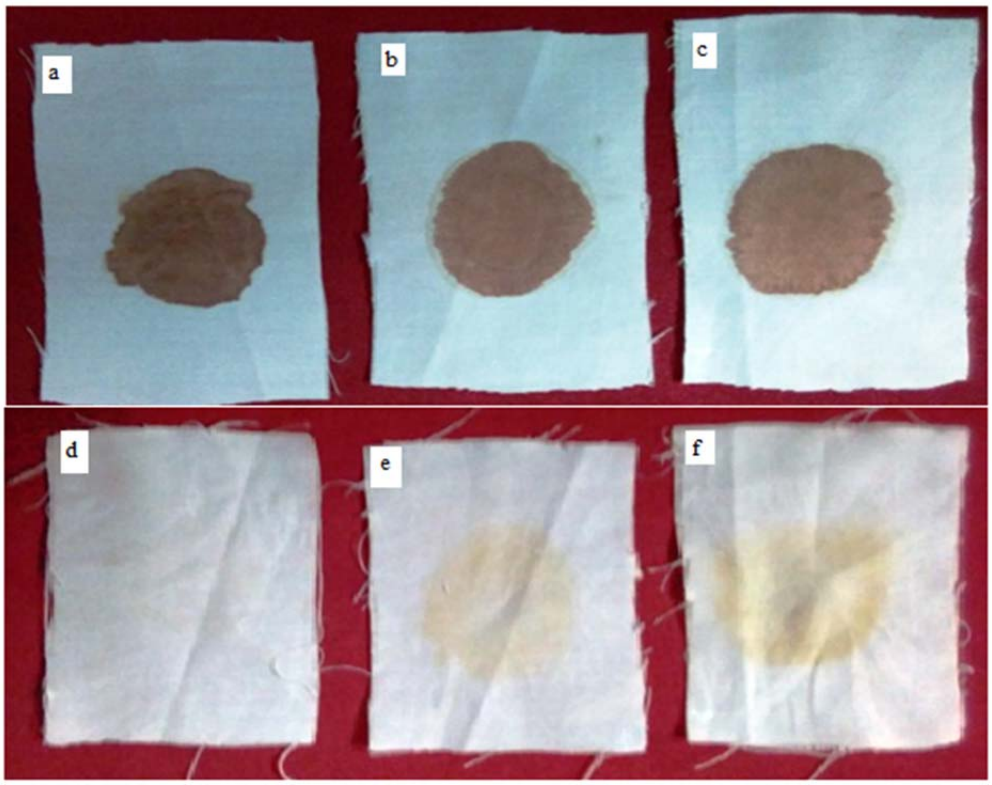

Figure 6. Determination of fibrynolytic potential of partially purified alkaline protease from *A. niger* DEF 1. a, b and c denotes cotton fabrics stained with blood sample; d, e and f represents stained cotton fabrics washed separately with partially purified protease along with detergent, distilled water with detergent and distilled water respectively.

Table 5. Morphological and Microscopic characteristics of *A. niger* DEF 1 isolated from dairy form effluent.

Characteristics of *Aspergillus niger* DEF 1	
Morphological	
Surface colour	Dark brown to black spores
Reverse side of the colony	Without colour
Margins	Entire
Elevations	Umbonate
Growth	Rapid
Microscopic	
Hyphae	Branched septate
Conidiophore	
Length	300 µm
Diameter	8 µm
Vesicle	Globose
Conidia	50 µm
Length	Blackish brown
Diameter	3 µm
Ornamentation	Spiny exine
Phialides	Biseriate
Cleistothecia	Present

The morphological characters of the fungal isolate, DEF1 (*A. niger*) were determined by growing the fungus on Czepekdox agar medium and the microscopic characteristics were determined by spore staining using lacto phenol cotton blue.

2010; Vaishali and Jain, 2012).

pH of the fermentation medium is one of the most important factor affecting enzyme production as microbial cells have no mechanism to adjust their internal pH and hence alteration in optimum pH range results in poor growth of microbes and hence poor enzyme production (Bhattacharya et al., 2011). In the present study a pH of 10 was found to be optimum for protease production. Alkaline proteases were found to have wide range of applications in detergent industry. Alkaline protease production was also reported by Kalpana Devi et al. (2008), Oyeleke et al. (2010), Vaishali and Jain (2012) and Palanivel et al. (2013) from different *Aspergillus* sp. Sonia and Saksham (2015) reported an optimum pH of 9.0 for the protease produced by *Penicillium chrysogenum* isolated from the soil. An optimum pH of 8.0 and 7.0 was reported by Chandrasekaran et al. (2015) for proteases produced by *Aspergillus flavus* and *A. niger* respectively isolated from paddy soil. Radha et al. (2011) and Mukhtar and Ikram-Ul-Haq (2009) reported optimum acid protease production by *Aspergillus* sp.

Incubation temperature also plays an important role in enzyme production and growth of microbes can be activated at one temperature and inhibited at another. It also influences the synthesis and secretion of enzyme production by changing the physical properties of the cell membrane (Balaji et al., 2012). In the present study, an optimum protease production was obtained at 50°C. Hence it was a heat stable protease. Ibrahim et al. (2009) reported maximum cell growth at 50°C and enzyme production at 37°C for alkaline protease production by *Bacillus* sp. An optimum incubation temperature of 40°C (Coral et al., 2003) and 45°C (Kalpana Devi et al., 2008) was reported for different *A. niger.* Chandrasekaran et al. (2015) reported an optimum temperature of 30 and 35ºC for the proteases produced by *A. flavus* and *A. niger* respectively isolated from paddy soil. An optimum incubation temperature of 35°C was reported for alkaline protease produced by *P. chrysogenum* isolated from soil samples (Sonia and Saksham, 2015).

Optimization of incubation period is very much essential as organisms show considerable variation in enzyme production at different incubation periods (Kumar et al., 2012). In our study, a maximum protease production was found at 96 h. Similar finding was reported for alkaline protease production from *Aspergillus Versicolor* by Vaishali and Jain (2012). Malathi and Chakraborty (1991) reported an incubation period of 48 h for protease production by *A. flavus*. Enhanced extracellular protease production after 144 h of incubation period was reported by Oyeleke et al. (2010) for both *Aspergillus fumigatus* and *A. flavus* isolated from local rice husk dump sites. Highest alkaline protease production was reported after 168 h of incubation by *P. chrysogenum* (Sonia and Saksham, 2015).

The enzyme was found to be very effective in removing blood stains from fabrics, hence can be applicable in detergent formulations. Similar finding was reported for alkaline protease from *Bacillus* sp. by Mala and Srividya (2010). Palanivel et al. (2013) reported prompt fibrinolytic activity for partially purified alkaline protease from *Aspergillus* strain KH 17 isolated soil samples collected from Eastern Ghats, Kolli hills region. Our study elucidates the use of dairy form effluents as good sources for isolation of alkaline protease producing fungal sp. The high yield of enzyme at alkaline pH (10) also suggests that this organism can find application in detergent and textile industries. The study gains its importance as proteases have innumerous applications in different industries.

Conclusions

The results of the present study clearly demonstrate that dairy form effluents can be used as potential sources for isolating alkaline protease producing fungi. As proteases accounts for more than 60% of the global industrial enzyme market, the present study gains its importance in isolating heat stable, alkaline protease producing fungal isolate. The high yield and activity of the protease at alkaline pH (pH 10) besides its fibrinolytic potential suggests its important role in various industrial applications especially in detergent formulations.

Conflicts of Interests

The authors have not declared any conflict of interests.

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