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Degradation of complex casein polymer: Production and optimization of a novel serine metalloprotease from *Aspergillus niger* KIBGE-IB36

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ABSTRACT

Serine metalloproteases have substantial scientific interest due to their unique physicochemical properties and capability to degrade complex casein polymer. In this contemporary study, the production of protease was enhanced by optimizing various physicochemical parameters and one-variable at a time approach used under submerged fermentation conditions. Among four different previously isolated *Aspergillus* species, *Aspergillus niger* KIBGE-IB36 represented high protease titer along with casein hydrolyzing zone on casein agar plate. Maximum protease production was achieved at 30 °C after 5 days of cultivation period at pH-6.0. Glucose (2.5 g L^{-1}) and casein (2.5 g L^{-1}) augmented protease production within the growth medium. Further increment in protease production was attained when different combinations of nitrogen and mineral salts concentrations used. Peptone (20.0 g L^{-1}), yeast extract (0.50 g L^{-1}), $CaCl_2$ (0.10 g L^{-1}), $MgSO_4$ (0.10 g L^{-1}) and K_2HPO_4 (0.30 g L^{-1}) boosted the protease secretion. The current study is imperative for the large scale production of serine metalloprotease that also possess extraordinary prominence in food processing industries.

1. Introduction

Casein is the complex protein polymer and major constitute of milk protein (80%). It contains aggregates of micelle (α-, β-, and κ-casein) and colloidal phosphate calcium (CCP) which have exceptional nutritional qualities and functional properties in innumerable food industries (Horne, 2006). Since most of the natural proteins lack desirable functional properties, their modification is critically important for improving different properties such as solubility, antioxidant activity, and health benefits. Besides its health benefits, casein possess most dominant causal allergen and causes cow's milk allergy (CMA). To overcome this unfavorable characteristic of casein, a special mechanism is required for transformation of complex casein matrix into simplified protein end product. Proteases (EC 3.4.21.24) catalyze the peptide bonds present within the complex casein structure. Proteases are the third largest group of the industrial enzymes and account for 60–65% of international enzyme market (Johnvesly and Naik, 2001; Singh et al., 2011). Proteases are important catalytic agents owing to their extensive industrial applications. They have potential demand in meat processing industry (Wang et al., 2016, silver recovery and leather processing industry (Gupta et al., 2002). In the pharmaceutical industry, slow-release dosage forms are prepared with proteases as additives (Roy MK, Watanabe Y et al., 2000, Okuno K, Yabuta M et al., 2002). In cheese processing, protease accelerates the ripening stage and develops flavor and texture of cheese. Therefore, an increasing demand of proteolytic enzymes with specific properties, has challenged biotechnologists to explore new sources of proteases. Among different sources, microbial proteases are widely used in bioprocesses; as they can secret bulk amount of extracellular enzymes and genetic manipulation of microorganisms is much easier as compared to animals and plants. Protease biosynthesis from fungal strains belonging to the genera *Aspergillus* (Tunga et al., 2003), *Penicillium* (Chrzanowska et al., 1993) and *Rhizopus* (Farley and Ikasari, 1992) are widely reported. Among them, *Aspergillus species* are preferred as they are generally regarded as safe (GRAS) by Food and Drug Administration (FDA) and have extraordinary applicability in various food formulations synthesizing procedures and industrial biotechnological sectors. Filamentous fungi possess unique characteristics as compared to bacterial species in terms

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of stability and easy recovery of the industrially valuable extracellular products. On the other hand, it is well known that production of extracellular protease from *Aspergillus* species is prominently influenced by various physicochemical parameters such as carbon and nitrogen sources, pH of the fermentation broth, incubation temperature and time. Production of protease is mostly enhanced by specific nitrogen source when incorporated in the fermentation medium (Abidi et al., 2008; Johnvesly and Naik, 2001; Bazarzhapov, Lavrent'eva et al., 2006; Chellappan et al., 2006; Chi et al., 2007).

In the present study, previously isolated *Aspergillus niger* KIBGE-IB36 was selected for extracellular protease production on the basis of qualitative analysis. Various fermentation conditions were investigated in order to induce the culture for hyper production of protease. Different physical and chemical parameters were varied using one-variable-at-a-time approach under submerged fermentation process for prospective industrial use.

2. Material and methods

2.1. Protease producing Aspergillus strain

Aspergillus fumigatus KIBGE-IB33 [Genbank: KF905648], *Aspergillus flavus* KIBGE-IB34 [Genbank: KF905649], *Aspergillus terreus* KIBGE-IB35 [Genbank: KF905650] and *Aspergillus niger* KIBGE-36 [Genbank: KF905651] are the fungal strains that were investigated in the current study to attain maximum production of protease. These *Aspergillus* strains were previously isolated from vegetative field of Karachi, Pakistan and characterized on the basis of taxonomical and molecular identification using 18S rDNA sequence analysis (Pervez et al., 2015). Qualitative screening of all of the pure cultures of *Aspergillus* was performed on casein agar plates that contained (%): potato dextrose: 3.0, sodium casein salt: 0.5 and agar: 2.4. Casein agar plates were inoculated with each culture separately and incubated for 7 days at 30 °C. The *Aspergillus* strain which showed larger zone of casein hydrolysis on casein containing agar plate and produced high quantity of protease in fermentation broth was selected for further investigation.

2.2. Protease assay and total protein estimation

The protease activity was estimated by modified Anson method using casein (0.2%) as a substrate and tyrosine as a standard. In order to analyze the catalytic performance of protease, 0.5 ml of cell free filtrate (CFF) was incubated with 1.0 ml casein solution prepared in tris-HCl buffer (pH-5.0, 50.0 mM) and kept at 50 °C for 15 min. The reaction was stopped by adding 5.0 ml trichloroacetic acid solution (10%) and the reaction tubes were incubated further for 30 min at 37 °C. The casein precipitates were removed by Whatman® filter paper. The precipitates free filtrate (2.0 ml) was mixed with 5.0 ml sodium carbonate solution (500.0 mM) and 1.0 ml Folin reagent. The reaction mixture was further incubated in dark for 15 min at 37 °C (Anson, 1938). The enzymatic casein hydrolysis releases high quantity of tyrosine in the reaction mixture that react with Folin reagent and produce a measurable color. The color intensity of the released tyrosine product can be measured through spectrophotometer at 660 nm wavelength. One unit of protease is defined as "*the amount of protease required to release 1.0 μM of tyrosine per minute under standard assay conditions*". The total protein content of CFF was estimated through Lowry's method using bovine serum albumin as a standard protein (Lowry et al., 1951).

2.3. Selection of fermentation medium for hyper protease production

In this study, different reported media (Table 1) were initially investigated for extracellular protease production under submerged fermentation process. All the fermentation media were inoculated with a newly selected *A. niger* KIBGE-IB36 and incubated for 7 days at 30 °C. A thick mycelium bed and remaining particles were removed through filter paper. The spores of fungal strain were subsequently removed by centrifugation at 40,000 × *g* for 15 min at 4 °C. The fermentation medium in which *A. niger* KIBGE-IB36 showed higher protease secretion was selected and modified in later studies.

Table 1
Composition of previously reported medium used for protease production.

Media Components (gL^{-1})	Medium-1[a]	Medium-2[b]	Medium-3[c]	Medium-4[d]	Medium-5[e]
Casein	0.5	–	10.0	10.0	5.0
Peptone	0.5	20.0	–	–	25.0
Yeast extract	–	10.0	–	–	20.0
Malt extract	–	10.0	–	–	–
Glucose	2.0	60.0	–	0.3	10.0
Sucrose	–	–	30.0	–	–
K_2HPO_4	10.0	–	–	–	0.5
KH_2PO_4	–	5.0	1.0	1.0	–
NaCl	–	–	–	0.3	–
$MgCl_2.6H_2O$	–	–	–	–	–
$MgSO_4.7H_2O$	5.0	5.0	0.5	–	0.1
$FeSO_4.7H_2O$	0.1	0.1	0.01	–	–
$FeCl_3$	–	–	–	–	–
$CaCl_2.2H_2O$	–	–	–	0.4	0.1
$(NH_4)_2SO_4$	–	–	–	5.0	–
Na_2HPO_4	–	–	–	1.6	–
$ZnSO_4.7H_2O$	–	–	–	0.01	–
pH[f]	8.4	8.6	6.0	6.0	7.0

[a] Sandhya et al. (2005).
[b] Srinubabu et al. (2007).
[c] Oyeleke et al. (2010).
[d] Siala et al., 2012.
[e] Qadar et al., 2009.
[f] pH of all media was adjusted before sterilization.

2.4. Analysis of physicochemical components of selected fermentation medium for higher protease production

All of the chemical and physical constituents of selected fermentation medium were optimized in order to enhance the extracellular release of protease from *A. niger* KIBGE-IB36 by following stepwise variation procedure under submerged fermentation process.

2.5. Optimization of physical components of fermentation medium for higher protease production from A. niger KIBGE-IB36

2.5.1. Impact of incubation period on the extracellular protease production from A. niger KIBGE-IB36

The incubation time of a culture within the fermentation medium is a major factor that directly affects the production of any bio product. In the current study, extracellular secretion of protease was examined by incubating the *A. niger* KIBGE-IB36 in the production medium for different time intervals (1–7 days) at constant pH (6.0), temperature (30 °C), and substrate concentration (0.1%).

2.5.2. Impact of temperature on the extracellular protease production from A. niger KIBGE-IB36

The influence of incubation temperature ranging from 20 °C to 60 °C was investigated for the maximum production of protease from *A. niger* KIBGE-IB36 by keeping the other parameters constant (casein 0.1%, pH-6.0 and 5 days incubation period).

2.5.3. Effect of pH on the extracellular protease production from A. niger KIBGE-IB36

The influence of pH was investigated for maximum protease production from *A. niger* KIBGE-IB36. For this purpose, the fungal isolate was cultivated in fermentation medium having different pH values (4.0–9.0) and kept at 30 °C for 5 days.

2.6. Optimization of chemical components of fermentation medium for higher protease production from A. niger KIBGE-IB36

2.6.1. Effect of glucose concentration on the production of protease from A. niger KIBGE-IB36

The influence of glucose concentration was also examined on protease production from *A. niger* KIBGE-IB36. Fermentation medium was prepared by using different concentrations of glucose ranging from 0.0-10.0 g L^{-1} under optimized conditions (pH: 6.0, temperature: 30 °C for 5 days of fermentation period).

2.6.2. Effect of casein concentration on the production of protease from A. niger KIBGE-IB36

Casein is the complex protein polymer that plays a crucial role during the protease production from fungal species. The influence of different casein concentrations (0.0–20.0 g L^{-1}) was analyzed in order to improve the extracellular release of protease from *A. niger* KIBGE-IB36. All other fermentation conditions were kept constant (pH: 6.0, temperature: 30 °C for 5 days of fermentation period).

2.6.3. Effect of different nitrogen sources on the production of protease from A. niger KIBGE-IB36

The effect of different nitrogen sources on protease production from *A. niger* KIBGE-IB36 was also observed by growing the fungal isolate in the production medium supplemented with yeast extract (0.0–5.0 g L^{-1}) and peptone (0.0–25.0 g L^{-1}).

2.6.4. Effect of micronutrients on the production of protease from A. niger KIBGE-IB36

The effect of different concentrations of salts on protease production was investigated by growing *A. niger* KIBGE-IB36 in the medium flasks that contained various salts with different concentrations ($CaCl_2$, 0.0–1.0 g L^{-1}; K_2HPO_4, 0.0–0.5 g L^{-1}; $MgSO_4$, 0.0–1.0 g L^{-1}).

3. Results and discussion

3.1. Screening and selection of fermentation medium for higher protease production

Four different previously isolated *Aspergillus* strains were screened quantitatively and qualitatively for extracellular protease production. Among them, *A. niger* KIBGE-IB36 [Genbank: KF905651] was capable to release high quantity of protease (33.0 U mg^{-1}) into the fermentation medium (Fig. 1a) and selected for further studies. A clear proteolytic zone around the mycelium growth of *A. niger* KIBGE-IB36 was also noticed on the casein containing growth medium (Fig. 1b). Selection of suitable nutrients is an essential parameter in order to improve cell proliferation and production of metabolites such as enzymes. Therefore, in the present study five different reported medium were carefully chosen for protease secretion from *A. niger* KIBGE-IB36. Medium 5 showed the highest production of extracellular protease from *A. niger* KIBGE-IB36 (33.0 U mg^{-1}) as compared to other reported media (Fig. 1c).

3.2. Optimization of physical components of fermentation medium for higher protease production from A. niger KIBGE-IB36

Certain time duration is required to microbial strain in order to produce high quantity of enzymes. Therefore, a wide range of different time intervals from 1-7 days were inspected in the current study to monitor the enzyme titer. The maximum protease production was achieved just after the late exponential phase and a sharp decline in protease activity was noticed after 5 days (Fig. 2a). This decrease in protease production might be due to the depletion of nutrients or change in the medium pH which consequently inhibit the production of enzyme. Enzyme activity depends on the fermentation time which may vary according to microbial species. Mostly, fungal enzymes are produced within 3–7 days of fermentation but in some cases this duration can be extended up to 15 days (Kaur et al., 2004).

The influence of temperature on protease production from *A. niger* KIBGE-IB36 was studied by performing the fermentation process at wide range of temperatures (20–60 °C). A gradual increase in protease production was noticed as the fermentation temperature raised and maximum enzyme production (58.0 U mg^{-1}) attained at 30 °C (Fig. 2b). However, further increase in temperature decreased the enzyme production. Approximately 55% decline in protease production was observed at 60 °C. Different microbial species exhibit different optimum temperature for their growth and metabolites production. The enzyme production may be affected by high temperature which consequently denatures the catalytic proteins and affects the metabolic pathways that eventually declines the product synthesis (Vieille and Zeikus, 2001). Various fungal strains showed maximum enzyme production at lower temperature values. It has reported that the increased alkaline protease synthesis from *A. tamarii* was achieved at 30 °C (Anandan et al., 2007). Additionally, haloalkaline serine protease from *Bacillus iranesis* (X5B) showed maximum enzyme production at 35 °C (Ghafoori et al., 2016).

The pH of fermentation medium affects the growth and metabolic regulation of the microbial cells; as microorganisms are sensitive to concentration of hydrogen ions which are present in the production medium. The pH of fermented medium also determines the nature of end product i.e. acidic, alkaline or neutral which further contributes to its commercial application at industrial level. The influence of initial pH of fermentation medium on the production of protease was examined by incubating the *A. niger* KIBGE-IB36 in fermentation medium having different pH range (4.0–9.0). It was found that the maximum protease production achieved when the medium pH was kept at 6.0 (Fig. 2c). Production of protease from *A. niger* KIBGE-IB36 decreased more than 25% when the initial pH of fermentation medium kept highly acidic (pH-4.0) or alkaline (pH-9.0). It may be due to the hydrogen ions concentration in the fermentation medium that regulates the multiplication of microbes and synthesis of enzymes which is one of the important factors at industrial processes (Raol et al., 2015; Wang et al., 2016). Similarly, alkaline protease from *Botrytis cinerea* revealed maximum enzyme production at pH-6.5 at 28 °C (Abidi et al., 2008; Anandan et al., 2007). It has been reported that *A. fumigates* RSP-8 and *B. halodurans* PPKS-2 showed high production yield of xylanase when medium pH was kept at pH-7.0 and pH-11.0, respectively (Vijayaraghavan and VInCEnT, 2012).

3.3. Optimization of chemical components of fermentation medium for higher protease production from A. niger KIBGE-IB36

The influence of various concentrations of glucose (0.0–10.0 g L^{-1}) was also examined for high production of protease in the fermented medium. It was observed that maximum production of protease was attained with 2.5 g L^{-1} concentration of glucose (Fig. 3a). It was noticed that beyond 2.5 g L^{-1} glucose concentration the synthesis of protease repressed. This might be due to the formation of excessive secondary metabolites that ultimately effect the protease production or due to the catabolic repression which might occurred during enzyme production in the medium. Protease synthesis was not attained from *A. niger* KIBGE-IB36 in the absence of glucose in the fermentation medium, it may be due to the fact that microbial cells might require carbon source for the cell multiplication and synthesis of products. It has been reported that *A. niger* produces alkaline protease in the presence of glucose (Dubey et al., 2010).

Filamentous fungal strains produce proteolytic enzyme in the presences of proteinaceous substrates added to the fermentation medium. Such substrates induce the microbial species in order to release high amount of extracellular proteases in the medium (Bazarzhapov, Lavrent'eva et al., 2006). Therefore, in this study different

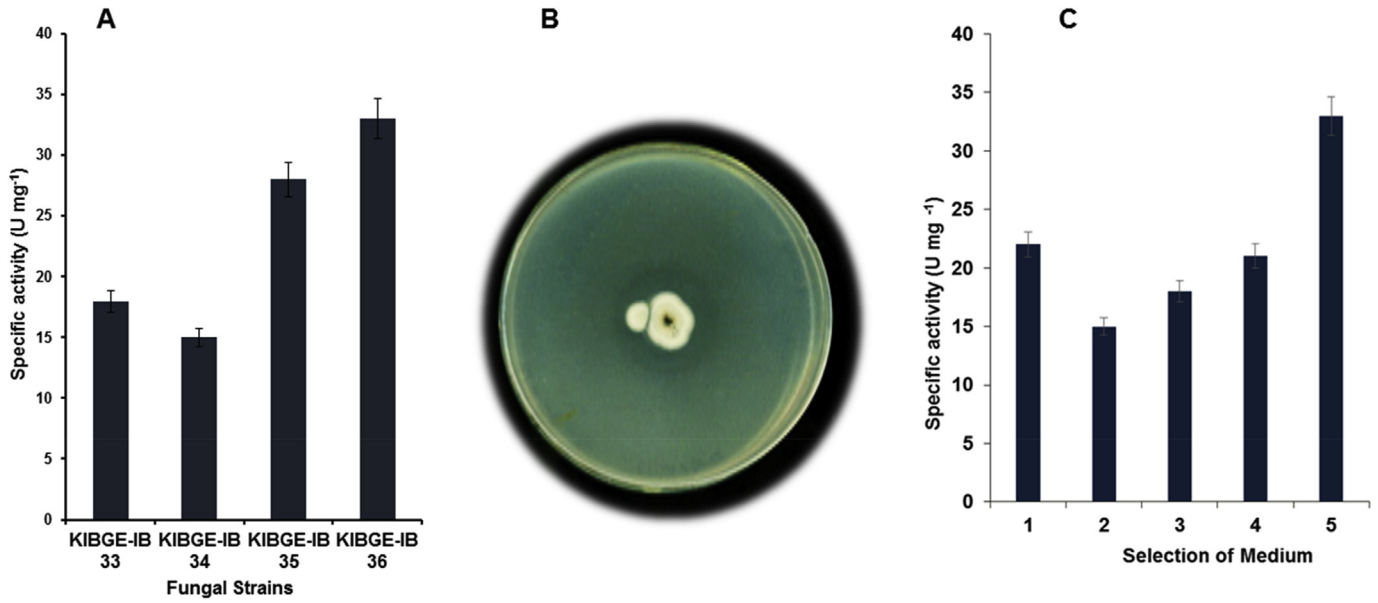

Fig. 1. (a) Production of protease by different *Aspergillus* species; (b) Casein hydrolytic zone around colony of *A. niger* KIBGE-IB36; (c) Selection of fermentaion medium for maximum prototease production. The values are shown as mean of three independent experiments (n = 3; Mean ± SD; p-value < 0.005).

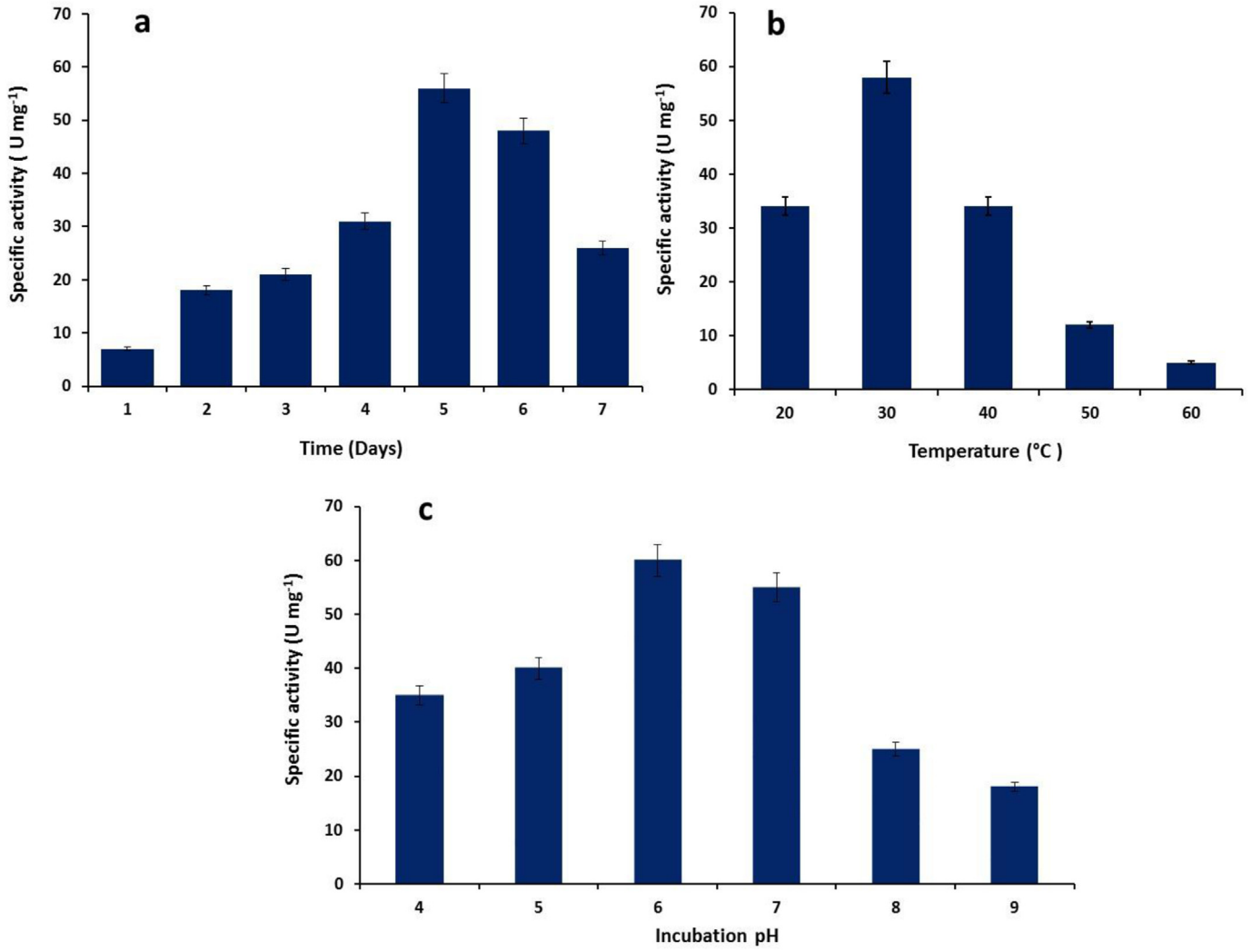

Fig. 2. Optimization of physical parameters for production of protease from *A. niger* KIBGE-IB36 (a) Fermentation time; (b) Incubation temperature; (c) Fermentation pH. The values are shown as mean of three independent experiments (n = 3; Mean ± SD; p-value < 0.005).

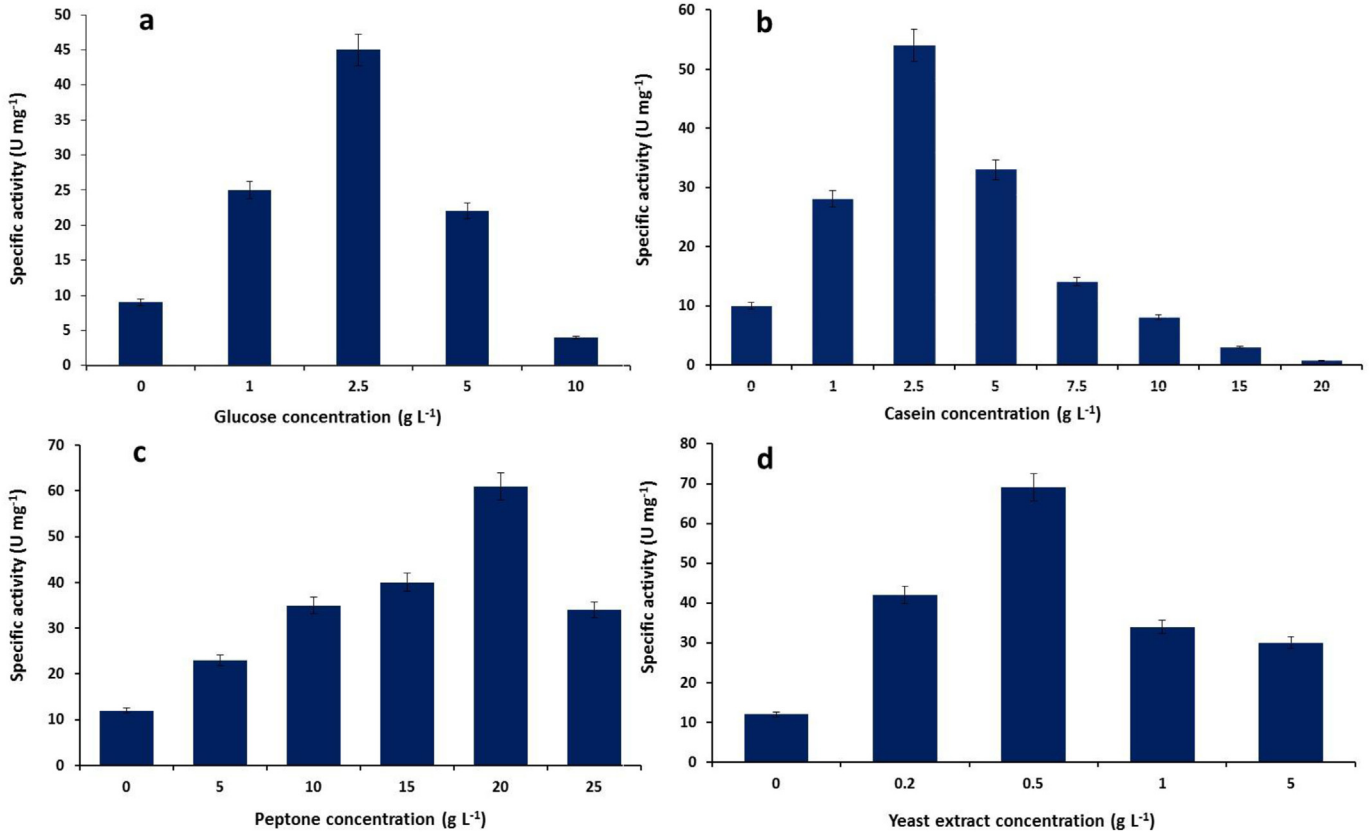

Fig. 3. Optimization of chemical components for production of protease from *A. niger* KIBGE-IB36; (a) Glucose concentration; (b) Casein concentration; (c) Peptone concentration; (d) yeast extract concentration. The values are shown as mean of three independent experiments (n = 3; Mean ± SD; p-value < 0.005).

concentrations of casein was investigated to produce maximum quantity of protease from *A. niger* KIBGE-IB36. It was found that protease production increased with the increase of casein concentration and maximum production was achieved at 2.5 g L^{-1} concentration of casein (Fig. 3b). However, beyond optimal level, the production of protease gradually declined by further increasing the concentration of casein. This might be due to the saturation of substrate in the production medium that inhibits the enzyme synthesis. However, in some other studies, casein or gelatin as an additional protein did not significantly increase the protease production.

Nitrogen source is one of the primary essential component that affects enzyme secretion from microbial species (Mehta et al., 2006). Therefore, considering its importance, various organic and inorganic nitrogen sources were investigated to improve the production of protease from *A.niger* KIBGE-IB36. Initially, the influence of different concentrations of peptone (0.0–25.0 g L^{-1}) and yeast extract (0.0–5.0 g L^{-1}) was studied under constant fermentation conditions. The maximum protease production (61.0 U mg^{-1}) was attained at 20.0 g L^{-1} concentration of peptone (Fig. 3c). However, minimal protease synthesis observed when the fermented medium was not supplemented with peptone. This might be due to the fact that peptone is the vital nitrogen source which is required for cell division and propagation. In addition, yeast extract contains essential ingredients such as vitamins, minerals and amino acids which are necessary for the growth of microbial cells and enzyme production (Sørensen and Sondergaard, 2014). It was noticed that 0.5 g L^{-1} concentration of yeast extract maximized the protease production and showed 78.0 U mg^{-1} specific activity (Fig. 3d). Further increase in the concentration of yeast extract, decreased the protease production which might be due to the alteration in hydrophobicity of microbial cell wall surface as it was reported that the hydrophobicity is associated with nitrogen sources and if the concentration of these sources changes, the hydrophobicity of the microbial surface will also affect. It is reported that when the peptone was added in the fermented medium followed by casein, the production of thermostable protease from *Penaeus vannamei* was increased in comparison to other nitrogen sources (Datta et al., 2017).

Micronutrients play critical role in maintaining the osmotic pressure of production medium. It is reported that appropriate concentrations of trace elements are essential for the substrate utilization of microbes (Luo et al., 2010). Therefore, in the current study, the influence of different salts such as K_2HPO_4, $CaCl_2.H_2O$ and $MgSO_4$ was analyzed on protease production from *A. niger* KIBGE-IB36. Concentrations of these salts were also investigated to select an appropriate concentration for maximum production yield of enzyme. It was found that 0.3 g L^{-1}, 0.1 g L^{-1} and 0.1 g L^{-1} concentration of K_2HPO_4, $CaCl_2$ and $MgSO_4$ was enough to generate optimum osmotic pressure for maximum protease production from *A. niger* KIBGE-IB36, respectively (Fig. 4). It was observed that as the concentration of these salts ions varied enzyme production varied and at hypertonic state, the water molecules flows out from microbial cells wall through osmosis and causes the cell wall to shrink which ultimately decreases the production of enzyme. Similarly at hypotonic condition (low concentration of salts in the fermentation medium) the water penetrates in the microbial cells and causes the cells to swell or burst. Different optimum concentrations of K_2HPO_4 have been reported earlier for various enzymes production from different *Aspergillus species*. *A. nidulans* required 0.3 g L^{-1} of K_2HPO_4 for maximum extracellular release of Beta-galactosidase (Kamran et al., 2017). The optimized concentrations of K_2HPO_4, $MgSO_4$ and $CaCl_2$ generate ideal osmotic pressure for the growth of *A. niger* KIBGE-IB36 in order to synthesize protease.

3.4. Group classification of proteases

The efficiency of enzyme-substrate based reactions generally effects

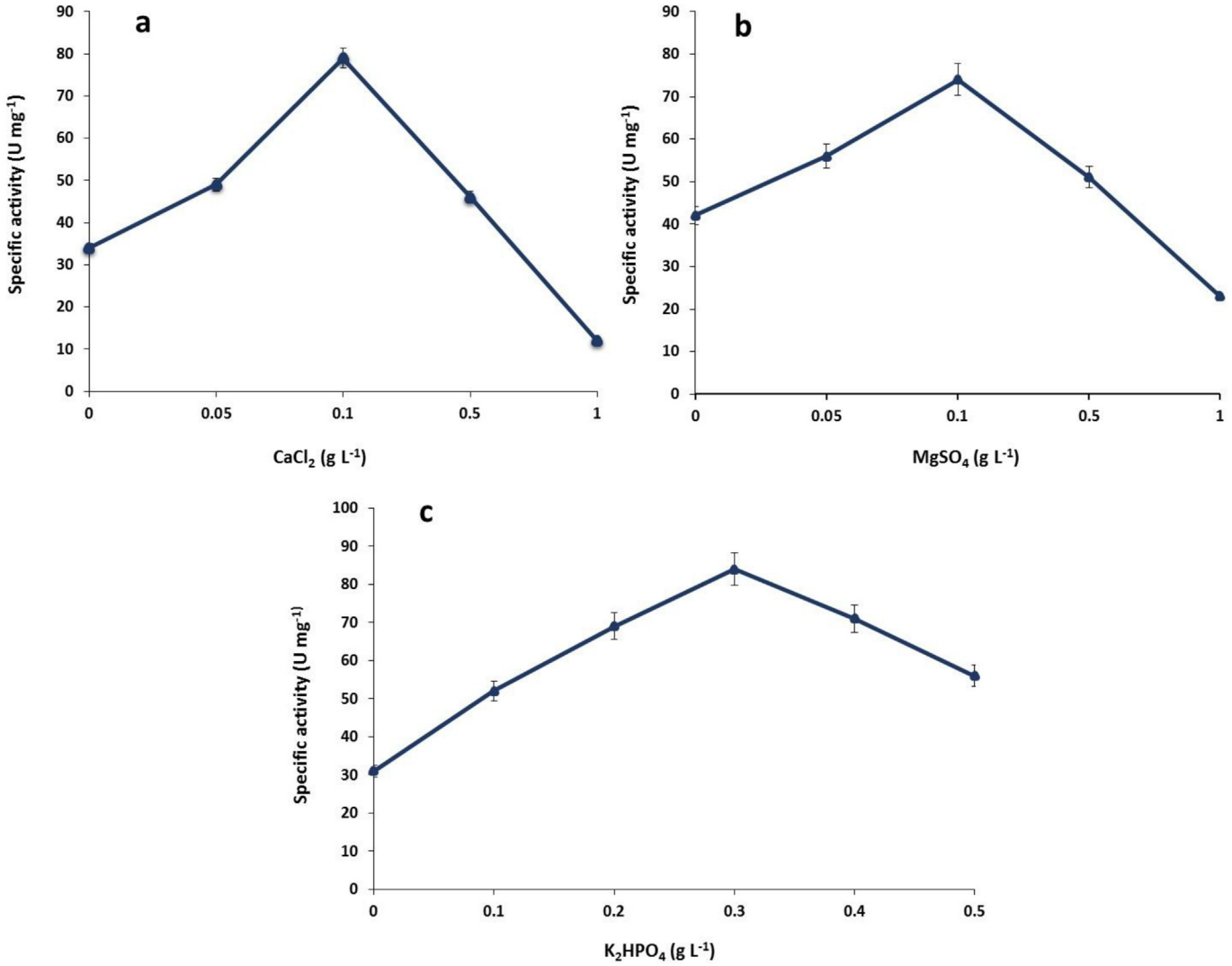

Fig. 4. Effect of micronutrients on protease production from *A. niger* KIBGE-IB36. (a) $CaCl_2$ concentration; (b) $MgSO_4$ concentration (c) K_2HPO_4 concentration. The values are shown as mean of three independent experiments (n = 3; Mean ± SD; p-value < 0.005).

due to different concentrations of metal ions which may activate or inhibit the catalytic mechanism of enzymes. In the current study, group classification of protease was investigated by inhibitory effect. After partial purification of protease through salt precipitation method, it was incubated with 5.0 mM metal ions chelator (EDTA) and PMSF (serine inhibitor) for different time durations (60.0 min and 120.0 min). The results suggested that protease from *A. niger* KIBGE-IB36 belongs to serine family of metalloprotease (Sattar et al., 2017).

3.5. Continuous degradation of complex casein polymer by serine metalloprotease

The serine metalloprotease obtained from *A. niger* KIBGE-IB36 also possess applicability in enzyme immobilization technology. The partially purified protease from *A. niger* KIBGE-IB36 was entrapped within polyacrylamide and agar-agar support matrices for continuous degradation of complex casein polymer. This enzyme showed incredible characteristics which have potential application in different sectors of food biotechnological industries (Sattar et al., 2018a,b).

In the current study, *A. niger* KIBGE-IB36 was investigated for maximum production of protease via submerged fermentation. Production of casein hydrolyzing protease indicated its significant utility in different industrial processes with short fermentation time at 30 °C. The optimization of different physical and chemical components including casein, glucose and nitrogen sources further augmented the protease production. From these findings it has concluded that the *A. niger* KIBGE-IB36 is the best microbial source for hyper production of protease which can be utilized in different industrial applications.

Disclosure statement

The publication of this manuscript is approved by all authors and they do not have any conflict of interest regarding any financial, personal or other relationships with any other people or organizations.

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