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Production and characterization of a thermostable extracellular esterase from *Aspergillus niger*

Producción y caracterización de una esterasa extracelular termoestable de *Aspergillus niger*

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Abstract

A strain of *Aspergillus niger* NRRL 337 degrade agro-industrial waste i.e wheat bran in solid state fermentation and produce esterase enzyme. Maximum enzyme activity 15.2 U/ml was produced after 72 h of incubation at 30ºC. The protein was partially purified by performing ammonium sulfate precipitation and immobilized metal ion chromatography (IMAC) technique. SDS-PAGE analysis showed molecular weight of esterase was 70 kDa. Effects of different nitrogrn and carbon sources with different concentrations were also studied. Partially purified enzyme was active at pH of 6.0 for up to 3 h and at temperature of 70ºC for 1 h. Ca^{2+}, Mg^{2+}, Na^{+1}, and K^{+1}play an important role in increasing enzyme activity. The residual activity of esterase was decreased in the presence of organic solvents. SDS, β-mercaptoethanol, and DMSO are good inhibitors for esterase enzyme. Under these conditions, partially purified esterase with specific activity of 114.20 U/mg and 47.58 purification fold was obtained. The data achieved from this work are important in terms of heat stability and pH of esterase and giving a new enzyme source for industrial applications.

Keywords: Esterase, *Aspergillus niger*, fermentation, thermostability.

Resumen

Una cepa de *Aspergillus niger* NRRL 337 degrada los desechos agroindustriales, es decir, el salvado de trigo en estado sólido de fermentación y produce la enzima esterasa. Se produjo una actividad enzimática máxima de 15.2 U/ml después de 72 h de incubación a 30ºC. La proteína se purificó parcialmente realizando una precipitación con sulfato de amonio y una técnica de cromatografía de iones metálicos inmovilizados (IMAC). El análisis SDS-PAGE mostró que el peso molecular de la esterasa era de 70 kDa. También se estudiaron los efectos de diferentes fuentes de nitrógeno y carbono con diferentes concentraciones. La enzima parcialmente purificada estuvo activa a un pH de 6,0 durante un máximo de 3 horas ya una temperatura de 70ºC durante 1 hora. Ca^{2+}, Mg^{2+}, Na^{+1}, y K^{+1} juegan un papel importante en el aumento de la actividad enzimática. La actividad residual de la esterasa se redujo en presencia de disolventes orgánicos. SDS, β-mercaptoetanol y DMSO son buenos inhibidores de la enzima esterasa. En estas condiciones, se obtuvo esterasa parcialmente purificada con actividad específica de 114.20 U/mg y 47.58 veces de purificación. Los datos obtenidos de este trabajo son importantes en términos de estabilidad térmica y pH de la esterasa y proporcionan una nueva fuente de enzimas para aplicaciones industriales.

Palabras clave: Esterasa, *Aspergillus niger*, fermentación, termoestabilidad.

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1 Introduction

Esterases (E.C 3.1.1) belong to hydrolases class of enzymes that catalyze the breakdown and formation of ester bond (Gopalan *et al*., 2015). Esterases mostly catalyzes the ester bonds upon hydrolysis of small chain esters or triglycerides that having carbon atoms less than 10. Most of the esterases keep the α/β hysrolase folds, which having a domain consists of a core of eight, mostly parallel β-sheet enclosed by α-helices. The active site generally composed of a consensus sequence Gly-X-Ser-X-Gly (where X is an amino acid) (Bornscheuer *et al*., 2002; Dilokpimol *et al*., 2017).

The process of action centers on the serine residues, its nucleophilic oxygen makes a tetrahedral hemiacetal intermediate with the substrate. The hydrolysis of the ester bonds produces the diacylglycerol and the enzyme is improved through the hydrolysis of the acyl group esterified with the serine residues (Sayali *et al*., 2013). In the existance of anhydrous organic solvent system, esterases catalyze the esterification, transesterification, and interesterification. Moreover, they usually show enantioselective, chemioselective and regioselective properties (Castro *et al*., 2018).

Esterases are present in microorganisms, animals and plants. A number of esterase was produced, purified, and characterized from microbial, plant, and animal sources. Different classes of esterases are found in literature like, cholineesterase, carboxylesterase, aryl esterase, acetyl xylan esterase, phenolic esterase, phosphotriesterase, tannin esterase, acetylcholinesterase, pectin esterase, and feruloyl esterase (Gupta *et al*., 2004; Nishimura *et al*., 2002).

Solid-state fermentation comprising of microorganisms and various wastes have attained better outcomes having low costs of enzyme production (Martins *et al*., 2008; Colla et *al*., 2010). Filamentous fungi is appropriate for processes i.e. solid state fermentation because of their capability to grow having a little volume of free water (Castiglioni *et al*., 2009; Thomas *et al*., 2013) and with their efficacy to reduce some pollutants (Ye *at al*., 2011). Mostly, the lipolytic enzyme production through the filamentous fungi in solid state fermentation consuming agro-industrial wastes like wheat bran has concerned much interest, since such enzymes could be extracted easily through fermentation medium because these are generally extracellular (Roveda *et al*., 2010; Hou *et al*., 2020).

Esterases are used in animal feed to enhance digestibility (Bonzom *et al*., 2018) bio-bleaching of wood-pulp, converting lignocellulosic substances to biofuels and feedstock and processing food to enhance clarification in fruit juices (Correa *et al*., 2013). These are also used in beverage production (beer and wine), and in synthesis of optically pure compounds, perfumes, and antioxidants (Topakas *et al*., 2007). It has been reported that esterases also helped in reduction of industrial pollutants and breakdown of natural materials like plastics, toxic chemicals, and cereal wastes (Panda *et al*., 2005). Carboxyl esterase is being used as biocatalyst in the preparation of cephalosporin derivatives (Doran-Pterson *et al*., 2008). Esterases are used in leather, textile, and paper industry (Liu *et al*., 2013).

In the present study, production and characterization of esterase from *A. niger* was studied taking into account various parameters for use in fruit juices and biofuel industries.

2 Methodology

2.1 Maintenance of fungal strain

Aspergillus niger NRRL 337 strain was acquired from culture bank of Institute of Industrial Biotechnology; Government College University Lahore, Pakistan. For maintenance of *Aspergillus niger* strain, potato dextrose agar (PDA) slants and plates were used.

2.2 Inoculum preparation

Inoculum was ready after addition of 10 ml double distilled water into a test tube having *Aspergillus niger* culture. About seven days old spores of *A. niger* were scratched gently with the help of sterilized loop and 1 ml inoculum was transferred to each flask.

2.3 Enzyme production

For the production of esterase enzyme, 10 g wheat bran was added into each of 250 ml Erlenmeyer flasks. About 10 ml of mineral salt solution (0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.05% KCl, 0.001% $FeSO_4.2H_2O$, 0.3% $NaNO_3$, and, 0.1% (v/w) Tween-80) was used (Atta *et al*., 2011). 1 ml of inoculum was added and flasks were incubated for 72 h at 30ºC.

2.4 Enzyme extraction

About 50 ml of phosphate buffer (0.1 M solution; pH 6) was transferred into fermented solid medium (10 g wheat bran) in a flask that was kept at 30°C for 30 min in a shaking incubator with 200 rpm. The content was centrifuged for 10 min at 6000 rpm. The cell-free supernatant comprising of extracellular esterase enzyme was taken for esterase enzyme assay.

2.5 Enzyme assay

It was performed using the method of Colen *et al.* (2006) with some improvements based on the titration with 0.05 M NaOH. Esterase assay was carried out by addition of 2.4 ml of phosphate buffer (0.1 M; pH 6) and 100 μl ethyl acetate (substrate) into the test tubes by incubation at 30ºC for 5 min. Then, 2.5 ml esterase enzyme was added and incubated for 25 min, 3 drops of phenolphthalein indicator were supplied and titrated with 0.05 M NaOH till light pink color was appeared. A control was also run in parallel. The enzyme activity was measured as reported by (Cerqueira, 2007). One unit of esterase enzyme activity was narrated as the volume of enzyme that produces 1 μmol of fatty acid per min.

2.6 Optimization of esterase enzyme production

Different factors like inoculum size, amount of wheat bran, incubation time, temperature and various carbon and nitrogen sources were observed. Different amounts of inoculum (1 ml, 2 ml, 3 ml, 4 ml, and 5 ml), wheat bran (5, 10, 15, and 20 g), time intervals (24, 48, 72, 96, and 120 h), pH range (6.0, 7.0, 8.0), carbon sources like xylose, starch, fructose, sucrose, maltose, glucose, lactose, and different nitrogen sources like $NaNO_3$, malt extract, peptone, Urea, yeast extract, NH_4 NO_3, and $(NH_4)_2SO_4$ were observed. Concentration of maltose and urea were further optimized. Standard deviations were also calculated.

2.7 Protein estimation

Total protein valuation was carried out using the method of Bradford (1976). In 5 ml of Bradford reagent, 900 μl buffer (phosphate buffer 0.1 M, pH 6) and 100 μl of esterase enzyme was added. A control was also run parallel which have about 5 ml Bradford reagent and 1 ml of buffer. Absorbance was observed in a spectrophotometer at 595 nm. For protein estimation, BSA was used as standard.

2.8 Purification of esterase enzyme

2.8.1 Ammonium sulfate precipitation

Partial purification of esterase enzyme was achieved with gradual increase of ammonium sulfate concentration to attain desired percentage of fractionation. Different fractions were proceeded from 30-80%. After each step, enzyme activity of supernatant and in pellet was analyzed. The dialysis of ammonium sulfate precipitated protein was carried out against phosphate buffer (pH 6) to remove the salts by using dialysis membrane (14 kDa MWCO; Cellu Sep T4, Vo/cm=3.46 mL).

2.8.2 Immobilized Metal Ion Affinity Chromatography

Immobilized metal ion affinity chromatography (IMAC) was performed via ProtinoR Ni-TED Kit. The columns used in this technique are ProtinoRNi-IDA columns that were ready for purification, prepared using 1X LEW buffer having four bed volumes, and the column was permitted to drain through gravity. Partially purified enzyme about two milliliters of volumes was transferred to pre-prepared column and was permitted to drain through gravity. The column was washed using 1X LEW buffer having eight bed volumes and was again drained through gravity. At last, elution was done by 3X elution buffer of three bed volumes, and the column was permitted to drain by gravity and pooled in various eppendorf tubes. Purified esterase enzyme fractions were collected and used for SDS-PAGE analysis and enzyme assay.

2.9 Molecular weight determination

Molecular weight of partially purified esterase enzyme was calculated using SDS-PAGE by following the method of Laemmeli (1970) using pre-stained protein ladder (10-250 kDa).

2.10 Characterization of partially purified esterase enzyme

2.10.1 Effect of pH and temperature

pH stability of esterase was observed after incubating the enzyme at room temperature with various pH-buffers ranging from 4.0-9.0 for 1-3 h. Temperature stability of the partially purified esterase was measured

after enzyme incubation ranging from 40-80°C for 1-4 h. Residual activity of esterase was measured using standard enzyme assay. A control was also run in parallel.

2.10.2 Effect of metal ions

Impact of several divalent ions such as Ca^{2+}, Mg^{2+}, Cu^{2+}, EDTA, Fe^{2+}, Na^{+1},and K^{+1} on enzyme was examined by pre-incubating esterase with these metal ions (1-10 mM). All metal ions were pre-incubated with enzyme for 1 h at room temperature. Residual activity was determined by standard enzyme assay. A control was also run in parallel.

2.10.3 Effect of various organic solvent and detergents

Stability of partially purified esterase enzyme was observed in the existence of 10-30% organic solvents i.e., ethanol, n-butanol, methanol, isopropanol, and acetone for 1 h at room temperature. Impact of different detergents like DMSO, tween-20, β-mercaptoethanol, SDS and tween-80 on partially purified esterase was detected. The enzyme was pre-incubated with dissimilar concentrations (1-3%) of these inhibitors for 1 h at room temperature. Residual activity of esterase was calculated by standard enzyme assay.

3 Results

3.1 Production of esterase

A. niger was grown on specified medium using wheat bran as substrate and was incubated for 72 h at 30ºC. The medium was centrifuged and esterase activity was measured in extracellular sample. Esterase enzyme activity was determined to be 15.2 U/ml.

3.2 Optimization of esterase secretion from A. niger

3.2.1 Effect of inoculum size and wheat bran amount

Effect of inoculum size on esterase production was determined after inoculating the fermentation medium i.e. wheat bran with different amount of *A. niger* culture. Maximum esterase enzyme production (4.64 U/ml) was achieved when 1 ml inoculum was used as described in figure 1 a. However, esterase enzyme production was reduced to 2.51, 3.08, and 1.18 U/ml when 0.5, 1.5, and 2.0 ml inoculum was used respectively. On the other hand, maximum esterase was produced (7.31 U/ml) when wheat bran of about 10 g was used as a substrate (Figure 1 b). Enzyme production was significantly decreased to 3.17, 4.15, and 1.17 U/ml after applying of 5, 15, and 20 g wheat bran respectively.

3.2.2 Effect of incubation time and pH

Effect of incubation time on esterase production was determined after incubating the media at 30°C for various time periods. Maximum esterase enzyme (8.55 U/ml) was estimated after 72 h (Figure 1 c). Similarly, reasonable amount of esterase was produced (7.01 U/ml) after 96 h. Maximum esterase was produced (9.36 U/ml) when primary pH of the media was set as 6.0. The production of enzyme was reduced to 4.45, 3.14 U/ml at pH 7.0 and 8.0 respectively (Figure 1 d).

3.2.3 Selection of carbon and nitrogen source

Effects of carbon sources on esterase production were observed after addition of different carbon sources into media and incubate it at 30°C. Maximum esterase enzyme (11.06 U/ml) was produced when maltose was used as a carbon source (Figure 2 a). Similarly, effect of different nitrogen sources on production of esterase was also determined. Maximum esterase was produced (12.24 U/ml) when urea was supplied to media as nitrogen source. However, reduction in the enzyme production was observed after addition of several nitrogen and carbon sources (Figure 2 b).

3.2.4 Optimization of concentration of maltose and urea

As maltose and urea was selected as a necessary carbon and nitrogen sources for maximum production of esterase and effect of the diverse concentrations of these sources on the enzyme production was assessed. It was noted that maximum esterase enzyme (13.25 U/ml) was observed when 0.3% maltose was used as described in Figure 2 c and esterase enzyme (15.2 U/ml) was achieved when 0.2% urea was used as described in Figure 2 d). The production of enzyme was reduced to 5.31, 4.58 U/ml after applying of 0.4 and 0.5% maltose respectively (Figure 2 c). Similarly, esterase activity was decreased to 6.56, 4.13, and 2.09 U/ml when 0.3, 0.4, and 0.5% urea was used as optimized nitrogen sources as described in figure 1 d.

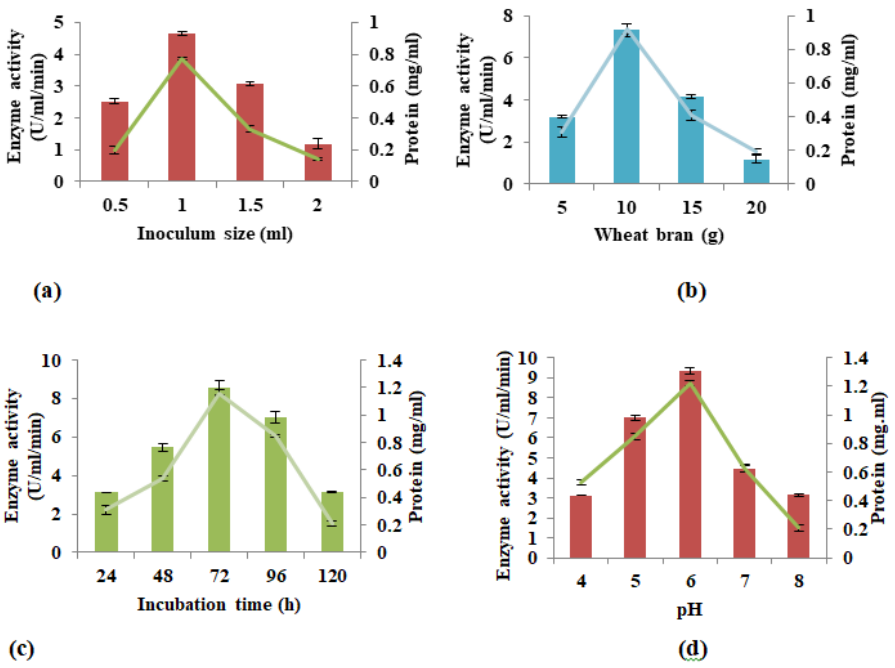

Fig. 1: Optimization of esterase enzyme production: (a) Inoculum size (ml) (b) Amount of wheat bran (c) Incubation time (d) pH.

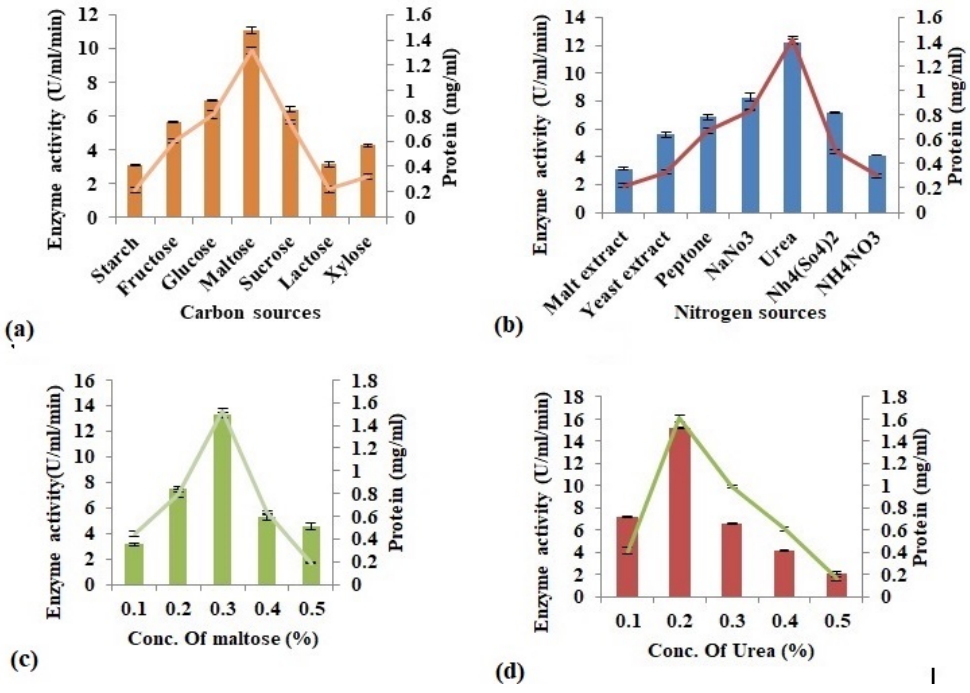

Fig. 2: Optimization of various parameters for esterase enzyme production: (a) Carbon sources (b) Nitrogen sources (c) Concentration of maltose (d) Concentration of urea.

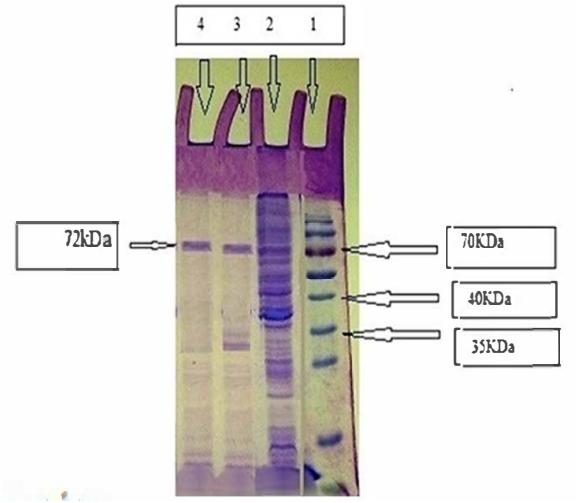

Fig. 3: SDS-PAGE Analysis: Lane 1: Protein marker, Lane 2: Crude sample, Lane 3: Ammonium sulfate fraction, Lane 4: purified esterase from *A. niger*.

3.3 Purification of Esterase

Crude esterase enzyme was partially purified through ammonium sulfate precipitation (50-70%). Partially purified aliquot with maximum esterase activity was further used in SDS-PAGE analysis. Esterase showed increased purification fold by 19.41 showing a specific activity of 46.59 U/mg. After immobilized metal ion affinity chromatography (IMAC), the esterase activity was reduced to 635 U. However, specific activity was determined to be 114.20 U/mg. The purification fold and percentage recovery was calculated to be 47.58 and 60.47% respectively as mentioned in table No 1.

3.4 SDS-PAGE Analysis

The purified fraction of the esterase enzyme was observed using SDS-PAGE (Figure 3). In lane 2, control sample without purification was run that showed the total protein profile of *A. niger*. In lane 4, a distinct single band of 70 kDa was observed indicating a somewhat high purity grade for the enzyme. In the fraction of partially purified esterase by ammonium sulphate precipitation method, apart from 70 kDa, some unwanted proteins were also present in lane 3.

3.5 Enzyme characterization

3.5.1 Thermostability

Residual activity of esterase enzyme was calculated by pre-incubating the enzyme at various temperatures (40-80°C) for 1-4 h. It was noted that esterase enzyme showed stability at 70°C for 1 h. Likewise, esterase displayed 76% residual activity at 70°C when incubated upto 4 h (Figure 4 a). Though, the enzyme activity was declined to 56% and 10% after 1 and 4 h of incubation at 80°C respectively. Esterase enzyme activity also revealed a sensible stability at 50°C and 60°C of about 94% and 92% residual activities after 1 h of incubation. Residual activity was significantly reduced at 80°C, to 36%, 25%, and 10% after 2, 3, and 4 h of incubation respectively.

3.5.2 pH stability

Residual activity of esterase enzyme was calculated by pre-incubation of enzyme with different pH buffers (citrate, glycine-NaOH and phosphate) for 1-3 h at room temperature. The residual activity of esterase was calculated by standard assay. It was observed that esterase was almost stable at pH 6.0 after 1 h of incubation. Likewise, the esterase showed 78% residual activity when incubated for 3 h at pH 6.0 (Figure 4 b). However, the esterase activity was declined to 42% and 19% after incubation of 1 and 3 h at pH 7.0 respectively. The enzyme revealed a fall in the stability at pH 8.0 with a residual activity of 22% after incubation of 1 h. At pH 4.0 and 5.0, residual activities were highly reduced respectively to 34% and 56% after 1 h of incubation.

3.5.3 Effect of metal ions

Enzyme was incubated with different concentrations (1-10 mM) of different metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+}, EDTA, Fe^{2+}, Na^{+1},and K^{+1}) to observe their effect on enzyme stability. It was determined that residual activity of the esterase enzyme was enhanced by 15% in the existance of 1 mM $CaCl_2$ as compared to untreated enzyme (control) (Figure 4 c). However, other metal ions effect on esterase was also observed. 1 mM EDTA solution was incubated with esterase enzyme ensued in reduction of residual activity (85%). Incubation of esterase enzyme with EDTA and high concentrations (5 mM and 10 mM) of metal ions cause inhibition in residual activity of the enzyme.

3.5.4 Effect of organic solvents

The effect of several concentrations (10-30%) of organic solvents i.e. acetone, absolute ethanol, methanol, isopropanol, and n-butanol was calculated after the esterase enzyme incubation with organic solvents for 1 h at room temperature.

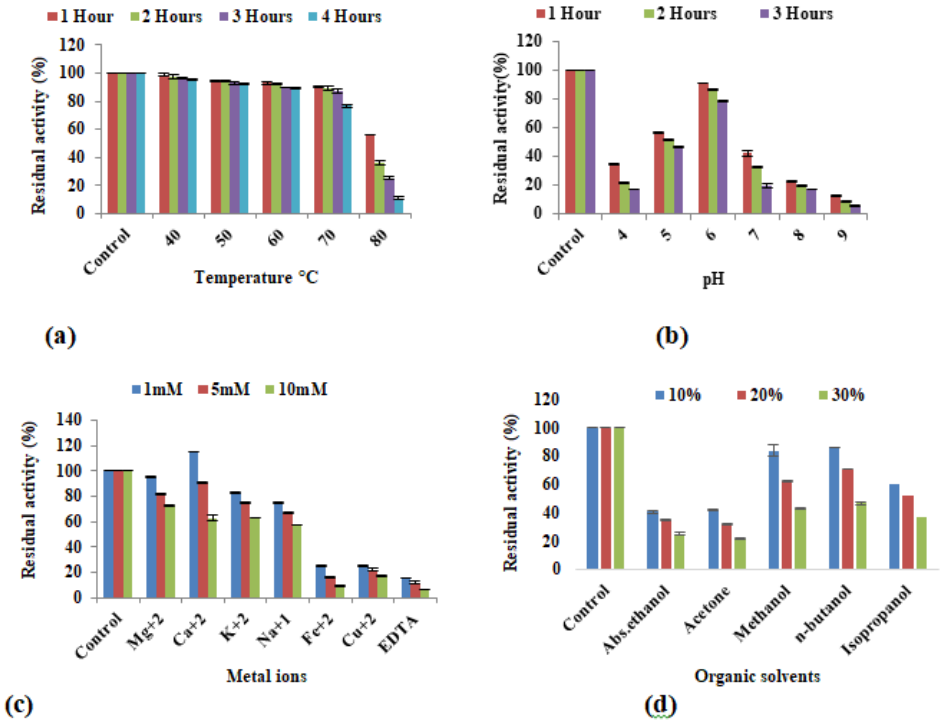

Fig. 4: Characterization: Efect of (a) Thermostability (b) pH (c) metal Ions (d) Organic solvents on the stability of esterase enzyme.

The result exhibited that the residual activity of esterase enzyme was 83%, 86%, and 60% at 10% concentration of methanol, n-butanol, and isopropanol. However at 30% concentration of absolute ethanol, and acetone was calculated as 25% and 21% of its residual activity (Figure 4 d).

3.5.5 Effect of detergents

The effect of various concentrations (1-3%) of detergents i.e. Tween-80, DMSO, SDS, and Tween-20 was determined after the esterase enzyme incubating with detergents for 1 h at room temperature. The outcomes exhibited that the residual activities of esterase enzyme was 65 and 72% in the presence of 1% Tween-20 and Tween-80 respectively (Figure 5). However, the residual activity of esterase enzyme after incubation with 3% concentration of Tween-20, SDS, DMSO, β-mercaptoethanol, and tween-80 was calculated as 48%, 7%, 17%, 23% and 47% respectively.

Discussion

In this study, esterase enzyme from *Aspergillus niger* was produced and partially characterized. The esterase enzyme activity of 15.2 U/ml was found after inoculation with 1 ml inoculum for 72 h at 30°C incubation. Esterase enzyme activity was further enhanced by optimizing the different factors for maximum enzyme production, for example, inoculum size, amount of wheat bran, time of incubation, pH, nitrogen, and carbon sources. Optimal temperature and time of incubation and esterase production was 30°C for 72 h respectively (Figure 1 c).). Camacho *et al.*, 2019 also observed the production of carbohydrate esterases from a *Aspergillus niger* grown on corn bran as substrate by solid state fermentation.

Maximum enzyme production was achieved with wheat bran of about 10 g of used as a substrate. It was because of the point that addition of specific quantity of wheat bran in the media sufficiently provides reasonable amount of nutrients (proteins 1.32%, carbohydrates 6.9%, fibers 2.6%, fats 1.9%, and 1.8% ash content composing of 0.05% Ca^{+2},

0.35% P, 0.17% Mg^{+2}, 0.12% S, 0.45% K^{+1}, and 0.23% several amino acids) necessary for proper microbial growth. These nutrients are important for growth of microbes and production of enzymes as stated by Park *et al*., 2002; Antonopoulou *et al*., 2018. Enzyme production was reduced when higher quantity of wheat bran was added in the fermentation medium. Our results were consistent with the results obtained by Teng *et al*. (2010) in which it was shown that higher concentration of wheat bran is the limiting factor in the production of esterase. Ou *et al*. (2011) stated the feruloyl esterase production from *A. niger* showed enzyme activity of about 7.68 U/ml using 10 g of wheat bran in solid state fermentation. However, increased quantity of wheat bran resulted in reduction in enzyme production. Our results are in agrrement to the findings of Ou *et al*. (2011) and low enzyme production due to increased quantity of wheat bran could be due to higher bed height or thickening of medium that created difficulty in the appropriate aeration. Sharma *et al*. (2020) also reported the production of ferulic acid esterase using the wheat bran as a substrate and found that it's a good source of nutrients. The appropriate air supply is important for best growth of mycelial hyphae and production of enzyme in fermentation medium (Lin *et al*., 2018; Abdullah *et al*., 2019). As Abdullah *et al*., 2019 use the *Aspergillus niger* in order to produce the enzyme via fermentation and this reference is given in relation to the fungi used for esterase production.

Maximum enzyme production was found after 72 h of incubation (Figure 1 c). As time of incubation increased, there is reduction in the quantity of nutrients in fermentation medium, increased inhibitor metabolites produced by fungi, age of fungi and consumption of sugar contents (Kanosh *et al*., 2001). Ramírez-Velasco *et al*., 2020 also stated that the maximum activity of chlorogenate esterase was produced by *Aspergillus ochraceus* via solid state fermentation after 96 h of incubation. Lipases just like esterases being lipolytic enzyme in nature Abdullah *et al*., 2018 also observed the production of lipase enzyme from Penicillium spp. after 72 h of fermentation. Lemos and Junior (2002) reported the maximum enzyme activity after inoculation of 144 h. So, this research work is important economically since decrease in the incubation time could reduce the cost of enzyme production. However, Meneses *et al*. (2020) reported 48 h of esterase production by *Aureobasidium pullulans* . Odinot *et al*. (2017) reported the esterase production by *Neolentinus lepidues* after 3 days of incubation.

The addition of mineral salt solution used to study the effect of divalent metal ions was necessary in maintaining the potential enzyme production.

To determine the optimal pH of the media, production of esterase enzyme was studied at different pH values. At pH 6, maximum esterase production was obtained (Figure 1 d. Production was decreased at pH 7.0 and pH 8.0. Atta *et al.* (2011) carried out the acetyl-xylan esterase production from *Penicillium notatum* at pH 5.0. In contrast, Sal *et al*. (2019) studied the expression of ferulic acid esterase (FAE) gene from *Geobacillus thermoglucosidasius* and reported optimal production at pH 8.5. pH of the media in which enzyme is produced affects the ionization state of the amino acids that determine the primary as well as secondary structure of enzyme and alter the activity of the enzyme. The effect of pH of fermentation media may be directly linked to stability of the enzyme.

The maximum production was obtained with maltose as a carbon source as described in figure 2 a. Carbon sources like maltose were metabolized and produced energy to induce microbial growth in enzyme production (Schneider *et al.,* 2018). Bhardwaj *et al.* (2020) also reported the better production of extracellular esterase from *Bacillus licheniformis* using 1% w/v galactose as a carbon source.

In current research work enzyme production was significantly increased in the presence of nitrogen source (urea) (Figure 2 b). When concentration of nitrogen source was increased, enzyme production was sharply decreased. Nitrogen source is mostly involved in protein synthesis of microbial metabolism. High concentration of nitrogen triggered toxicity that has an adverse effect on enzyme productivity and in the development of biomass. Our work is according to the work reported earlier by Seyis *et al*., 2003. Our findings are related to Kowalczyk *et al*. (2017) who demonstrated that addition of urea in medium has a profound effect on esterase production from *A. niger*

In second round of purification, esterase was purified by IMAC technique. The specific activity of the enzyme was increased to 114.20 U/mg and purification fold reached to 47.58-fold (Table 1). An intracellular esterase from *Bacillus aryabhattai* B8W22 was isolated and purified up to 59.03 purification fold with enhanced yield of 20% (Zhang *et al.,* 2019). Chen *et al*. (2016) described the purification of esterase enzyme and reported 8.93% yield, specific activity of 8121.51 U/mg and 9.33 purification fold.

In this study, SDS-PAGE was carried out to calculate molecular weight of esterase enzyme that came out to be 70 kDa (Figure 3). However, molecular

Table 1. Purification steps of esterase from A. niger

Purification steps	Esterase activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude enzyme	1050	425	2.4	1	100
$(NH_4)_2SO_4$ precipitation (70%)	725	15.56	46.59	19.41	69.04
IMAC	635	5.56	114.20	47.58	60.47

weight of 76 kDa of esterase produced from a marine fungus *Fusarium* sp. DMT-5-3 (Luo *et al*., 2015) and molecular mass of 60 kDA of carboxyl esterase from *Aspergillus nomius* HS-1 had been described (Uchida *et al.,* 2003).

Effect of thermostability on esterase enzyme showed 76% residual activity at 70°C after 4 h of incubation (Figure 4 a). For thermostable enzymes, thermostability is an intrinsic property and determined by the primary structure of protein.

The structure of enzyme at higher temperature changes because structure turns into extra flexible to expose the active sites for maximal binding to the substrate. The overall rigidity and active site flexibility are reflected to be adaptive as they promote both termal stability and enzymatic activity. (Kokkinidis *et al*., 2012; Xu *et al*., 2017). The geometric arrangement of the catalytic triad of the thermostable esterase is less affected at high temperature, representing the enzyme stable and active at high temperature for catalysis (Li *et al* ., 2012). Tang *et al*. (2019) reported glucuronoyl esterase from *Thielavia terrestris* and determined its thermostability at 55°C. A thermostable Type B feruloyl esterase was characterized from thermophilic *Thielavia terrestris* and enzyme showed maximum residual activity of about 96% and 84% at 50°C and 60°C after 1 h of incubation *(*Meng *et al*., 2019). Makela *et al*. (2018) had characterized a feruloyl esterase from *Aspergillus sterreus* that remained stable between 25-80°C; however it showed 80% of its residual activity at 50°C.

Effect of pH onto enzyme stability determined that the enzyme remained stable at pH 6.0 and showed 91.03% residual activity (Figure 4 b). However at other pH values, the enzyme showed reduced residual activity. Dilokpimol *et al*. (2017) showed stability of the feruloyl esterase enzyme at neutral pH. Zhang *et al*. (2015) also reported esterase enzyme that was active from pH 4.0 to 8.0.

Metal ions effect is very important as they act as a co-factor in some enzymes. Mg^{2+}, Ca^{2+}, Na^{+1}, and K^{1+} enhanced esterase activity when enzyme was pre-incubated with metal ions (10 mM) (Figure 4 c). Liu *et al*. (2013) determined the stability of fungal (*Rhizomucor meihi)* esterase towards Ca^{2+} and Mg^{2+}. Li *et al*. (2014) determined an inhibition of the enzyme with EDTA recommends that this esterase may want a divalent cation as a cofactor. Stability of esterase enzyme in the presence of Na^{+1} and K^{+1}may bebecause of salting in effect. An esterase produced from *Aspergillus* spp. showed the stability in the presence of $Ca^{2+}$and Mg^{2+} (Wu *et al*., 2018).

Esterase enzyme showed the stability towards methanol (83%), n-butanol (86%), and iso-propanol (60%) (Figure 4 d). The result of this study was comparable with the results obtained by Wu *et al*. (2016) in which it was revealed that esterase was stable when enzyme was pre-incubated with 30% iso-propanol, metanol, and n-butanol. Lemes *et al*. (2019) purified esterase from *Aureobasidium pullulans* and studied its stability towards methanol at high concentration (100%). Adigüzel. (2020) characterized a thermostable, halostable, and solvent stable esterase from *Bacillus mojavensis* and showed its stability in the presence of acetone, metanol, and ethanol. The capability of the esterase to endure water-resistant environment is important for its application in organic synthesis. The capability of partially purified esterase in existence of organic solvents suggested its potential applications in the biotechnological fields.

Surfactants can change the tertiary conformation of the enzymes that ultimately leads to the reduced residual activity of the enzyme. In the existence of SDS (ionic detergent) residual activity of esterase enzyme reduced as stated in the figure 5 that showed the hydrophobic interactions are significant in retaining structure of enzyme (Uchida *et al*., 2003). Tween-80 has the oleic acid composition in its nature and maintains the enzyme stability. As in literature it was reported that tween-80 helps the enzyme production and fungus growth when directly add into fermentation media (Castro *et al*., 2018). Addition of β-mercaptoethanol (1-3%v/v), disulfide bond-reducing agents, resulted in the stability of esterase activity, signifying that the enzyme did not have any important disulfide bond. The existence

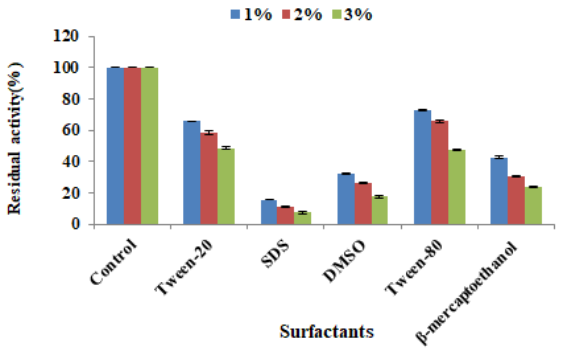

Fig. 5. Characterization: Effect of surfactants on the stability of esterase enzyme.

of these thiol group-protecting agents motivates the enzyme activity by inhibiting the oxidation of sulfhydryl groups (Ryu *et al*., 2016).

Conclusions

Agro-industrial waste i.e. wheat bran has been used as nitrogen and carbon sources for growth of microorganisms and production of esterase by *Aspergillus niger,* a fungus that belongs to a main genera of filamentous ascomycetes. The wheat bran demonstrated to be the most suitable medium for esterase production by solid-state fermentation. The data achieved from this research work are important in terms of heat stability and pH of esterase and presenting a new enzyme source for industrial applications. Also, these characteristics make esterase a potential candidate for biotechnological applications.

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