Production and Purification of Peroxidase from *Aspergillus niger.*

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Abstract

This study was conducted in the laboratories of Biology Department, College of Science, which deals with isolation and purification of peroxidase and optimization of process parameters to achieve maximum yield of peroxidase by *Aspergillus niger*. Solid-state fermentation of *Aspergillus niger* was carried out for enhanced production of peroxidase using hydrogen peroxide as the substrate of enzyme maximum activity of the enzyme was achieved under optimum growth conditions. The optimum conditions were the isolated of *Aspergillus niger* from soil and growth in synthetic medium, it gave high titer of peroxidase activity, the fructose as carbon source, peptone as nitrogen source, after 12 days of incubation, incubation temperature 25 °C and pH = 6.5. Peroxidase purified in four purification steps; precipitation with 70% saturation of ammonium sulfate, step of dialysis, the third by ion exchange chromatography using DEAE-Cellulose and fourth by gel filtration throughout Sephadex G-100.

The specific activity of the purified enzyme was 150U/mg with 7.75 folds. The peroxidase was shown to have molecular weight of 40kDa in SDS-PAGA and about 40kDa in gel filtration.The optimum pH and temperature for peroxidase activity 7 and 35 C^0 respectively.

Keywords:Peroxide, Aspergillus niger, Puripication, characterization

الخلاصة

اجريت هذه الدراسة في مختبرات جامعة بابل/كلية العلوم/قسم علوم الحياة و تضمنت استخلاص و تنقية انزيم البيروكسيديز وتحديد الظروف المثلى لإنتاج الانزيم من الفطر A.niger. تم تحفيز زيادة انتاج الفطر للإنزيم باستخدام المزارع الصلبة. كانت الظروف المثلى لعزل الفطر من الاوساط الصلبة و الاوساط التركيبية اعطت اعلى فعالية لأنزيم البيروكسيديز عندما استخدم الفركتوز كمصدر للكاربون و البيبتون كمصدر للنتروجين بعد 12 يوم من الحضن. كانت درجة حرارة الحضن 25 م ° عند رقم هيدروجين 6.5. نقي الانزيم بأربعة طرق تنقية : الترسيب بكبريتات الامونيوم بنسبة اشباع 70%, الديلزة, التبادل الايوني باستخدام المبادل الايوني DEAE.Cellulose و الترشيح الهلامي باستخدام سيفادكس جي− 100. اظهرت النتائج ان الفعالية النوعية للإنزيم المنقى 150 وحدة/ ملي غرام و عدد مرات التنقية 7.75 و كان الوزن الجزيئي 40كليو دالتون بالترشيح الهلامي و بالترحيل الكهربائي. و اظهر الانزيم اعلى فعالية في درجة حرارة 35 م ° ورقم هيدروجين 7.

الكلمات المفتاحية: انزيم البيروكسيديز , فطر A.niger, تنقية, التوصيف

Introduction

Aspergillus niger is a filamentous fungus belonging to phylum ascomycota, it produces microscopic conidia on conidiophores that are produced asexually, hyphae possess septa and are hyaline, they are supported at their base by foot cells from which conidiophores originate, it possesses long, double-walled, smooth and colorless to brown conidiophores, the major morphological distinction of *A. niger* from other species of *Aspergillus* is the presence of carbon black or dark brown spores on biseriate phalides, which are arranged in a globose head radiating from a vesicle conidiophoreson the basis of the importance of glucoamylase, the present study has been taken to isolation of glucoamylase and optimization of process parameters to achieve maximum yield of glucoamylase by *Aspergillus niger*. Many useful enzymes are produced using industrial fermentation of *A. niger,* for example, glucoamylase is used in the production of high fructose corn syrup, pectinases are used in cider and wine

clarification , Alpha-galactosidase, an enzyme that breaks down certain complex sugars, glucose oxidase (GOD), glucose oxidase is used in the design of glucose biosensors, due to its high affinity for (β-D-glucose), peroxidase, lipases, cellulose, xylanases, and protease (Schuster *et al*., 2002 ; Staiano *et al*., 2005; Immanuel *et al*., 2007; Rebecca *et al*., 2012). Peroxidase (EC1.11.1.13) as wood decayingbasidiomycetes. Peroxidases are extracellular glycoprotein produced only by ligninolytic (wood-rotting and litter-degrading), they catalyse the H_2O_2. The complex is a highly reactive oxidant that can freely diffuse away from the enzyme's active centre because of its low molecular weight. Hence, it non-specifically oxidizes a variety of phenolic and nonphenolic substances, including lignin and toxic pollutants (Steffen *et al*., 2000). By removing lignin, fungi are able to access plant polysaccharides (hemicelluloses, cellulose), which serve as their primary source of carbon and energy. Hence, these ligninolytic enzymesare used in various biotechnological applications in pulp and paper, food, textile and dye industries, bioremediation, cosmetics, analytic biochemistry and many others (Rogalski *et al*., 2006). On the basis of the importance of peroxidase, the present study has been taken to isolation and purification of peroxidase and optimization of process parameters to achieve maximum yield of peroxidase by *Aspergillus niger*.

2-Materials and Methods

2-1:Fungus isolates:

Aspergillus niger was isolated from different soils and orange fruits. The *Aspergillus* cultures were isolated from the soil by the serial dilution method of Clarke and Svensson (1984). For isolation of fungus from orange fruits by the method of King *et al* (1986)**.** The surface disinfected fruits were plated on sterilized petri dishes contain PDA. Then they were incubated at 28 ºC for seven days, pure cultures were maintained in PDA media at 4°C and were subcultured at 30 days interval. Identification of *Aspergillus* isolates was based on cell and colony morphology characteristics as per the method described by Raper and Fennel (1965).

2-2 : Inoculum preparation and Isolation of peroxidase

Pure culture of newly isolated *Aspergillus niger* was maintained on Potato Dextrose Agar (PDA) slants at 4 °C, the growth (synthetic) medium (Table 1) was autoclaved for 15 min, after cooling the flasks, inoculum was prepared by 10 ml of sterilized distilled water which was added to a sporulated 5 days old PDA slant culture, inoculum (5 ml) was added to each flask that containing 100 ml of growth medium with the help of sterilized disposable syringe and flasks was incubated at 22 °C on a rotary shaker at 160 rpm for 7 days. At the end of fermentation, the mycelium was separated from the culture broth by filtering through a filter paper (Whatman No.1), the filtrate was centrifuged at 5,000 rpm for 15 min at 4° C to remove the suspended particles, the supernatant was carefully collected and stored under refrigerated conditions as to crude enzyme for further purification steps after enzyme assay (Stajić *et al*., 2010) .

Table 1. Compositions of growth (synthetic) medium of *Aspergillus niger*.

S/N	Ingredients	Quantity(g/1000 ml)
1	glucose	10
2	yeast extract	2
3	NH_4No_3	0.2
4	$MgSO_4$. 7H2O	0.5
5	K_2HPO_4	1
6	$NaH_2PO_4.H_2O$	0.4
7	Distilled water Up to	1000 ml mark

2–3:Assay of peroxidase

peroxidase activity was assayed for by incubating 0.1 ml of the crude enzyme with 0.05 ml of 30% hydrogen peroxide , 0.05 ml of 0.018 M guaiacal and 2.80 ml of 0.1 M potassium phosphate buffer (pH= 8.6) at 25 ^{o}C for 5-6 min after incubation, the colour developed was indicated by measuring its optical density using a spectrophotometer at 436 nm. One enzyme activity unit (U) was defined as the amount of enzyme which catalyses the conversion of 1 µmol of hydrogen peroxide per minute at 25 ^{o}C (Bergmeyer, 1974) .

2-4 : Protein determination:

Protein content was measured according to Bradford (1976) figure (1).

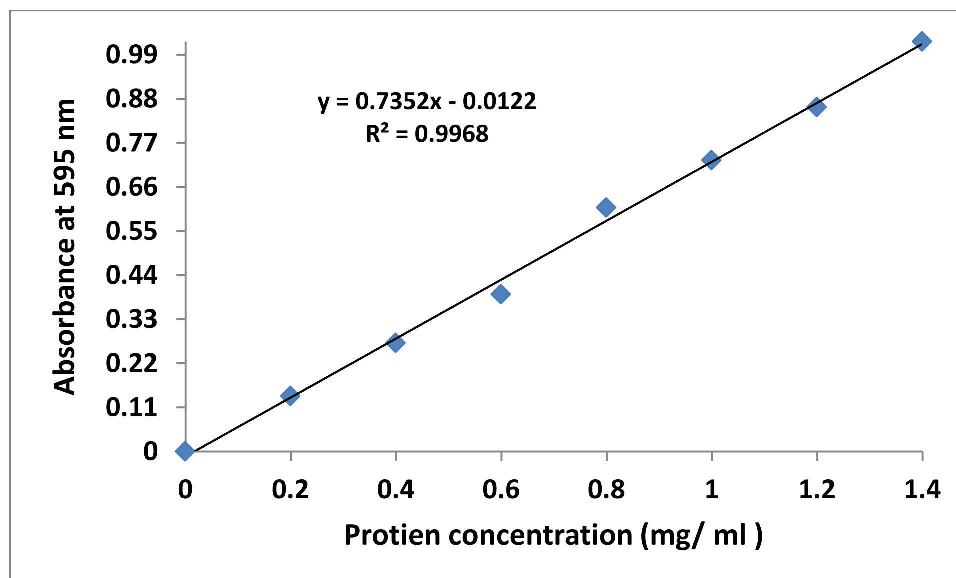


Figure (1). Standard curve for the different concentration of bovine serum albumin at 595 nm.

2-5 : Factors affecting peroxidase production (Johnsy and Kaviyarasan, 2011)

2-5-1 : Culture media : the optimum culture medium of the peroxidase production was determined by assaying activity using 30% hydrogen peroxide as the substrate of enzyme using different culture media include [synthetic medium, potato dextrose broth (PDB) and yeast extract broth (YEB)].

2-5-2: Incubation temperature : The optimum incubation temperature of the peroxidase production was determined by assaying activity using 30% hydrogen peroxide as the substrate of enzyme using different temperatures degree (20, 25, 30,35,40) °C.

2-5-3 :pH values: the optimum pH of the enzyme production was also determined by assaying activity using 30% hydrogen peroxide as the substrate of enzyme using different pH values (4, 4.5,5,5.5,6, 6.5,7, 7.5, 8, 8.5, 9) at 25 °C.

2-5-4 : Incubation periods : The reaction mixture was incubated at different incubation period (24, 48, 72, 96, and 120) hours at optimum pH(6.5) and 25 °C.

2-5-5 : Effect of different carbon sources on peroxidase production:

The effect of various carbon sources on peroxidase production was done as described by using the previously described production medium. The carbon sources studied are fructose, sucrose, mannitol, lactose, glucose, the pH of the medium was adjusted to (6.5) and cultures were incubated at 25 °C for 12 days, peroxidase activity was determined thereafter.

2-5-6 : Effect of different nitrogen sources on peroxidase production:

The effect of various nitrogen sources on peroxidase production was done as described by using the previously production medium. The nitrogen sources studied are beef extract, peptone, ammonium chloride, ammonium nitrate and ammonium tartarate, the pH of the medium was adjusted to 6.5 and cultures were incubated at 25 °C for 12 days , peroxidase activity was determined as previously described.

2-6. **Purification of crude peroxidase from *Aspergillus niger***

Purification of enzyme is done by four methods; ammonium sulphate precipitation method, dialysis, ion exchange and gel filtration chromatographic method.

2-7. **Electrophoresis**

PAGE was performed by the method of Laemmli (1970)

2-8**. Molecular mass determination**

The molecular weight of purified enzyme was estimated in gel filtration and SDS-PAGE

2-9.**Optimum PH**

The optimum pH value for the peroxidase activity was determined by assaying enzyme activity at different pH (3-11)

2-10. **Optimum temperature**

The optimum temperature value for the peroxidase activity was determined by assaying enzyme activity at different temperature (10-60)

3- Results and Discussion

3-1 : Optimum culture medium for peroxidase production

The over production of enzyme by *Aspergillus niger* was isolated from soil and growth in synthetic medium, it gave high titer of peroxidase activity (8.565 U/ml) followed by yeast extract broth (3.231 U/ml) while potato dextrose broth (PDB) gave low titer of peroxidase activity (0.847 U/ml) Fig. 2. The selection of the suitable fermentation medium is a critical factor for microbial growth and enzyme formation, the growth of an organism in culture medium is influenced by the nutrient composition of the medium and the availability of these nutrients (Ellaiah, *et al*., 2002). Other studies like Stajic *et al* (2011) used the same medium for production of peroxidase from *Trametes gibbosa,* also Batool *et al* (2013) used the synthetic medium for production of peroxidase from *Ganoderma lucidum* IBL-05.

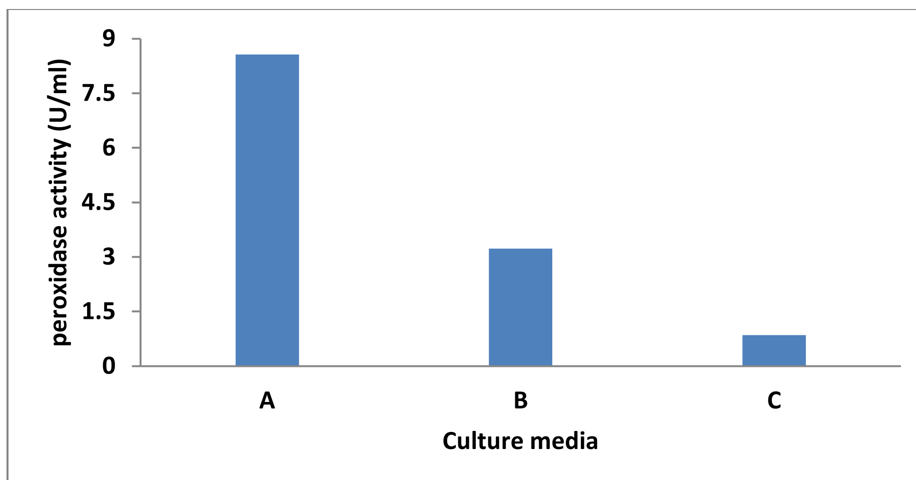


Figure (2) : Effect of culture media (A: synthetic medium, B:Yeast extract broth C: Potato dextrose broth) on the production of peroxidase from *A.niger*.

3-2 : Optimum incubation period for peroxidase production:

Figure (3) shown the increasing of enzyme activity with increasing the incubation period until reach to maximum activity (10.763 U/ml) after 12 days of incubation , then it began to decreased (5.221, 2.324, 0.062 U/ml) after 13,14,15 days of incubation respectively. Incubation period being an important parameter that has been controlled for optimum enzyme formation and it varies from organism to organism due to variation in the lag and log phases of growth (Bhatti *et al.*, 2007).

This study was agreed with Prasher and Chauhan (2013) when they shown the maximum activity of peroxidase from *Grammothele fuligo* achieved after 12 days of incubation , while Manimozhi and Kaviyarasan (2012) reported the highest activity of peroxidase from *Agaricus heterocystis* achieved after 17 days of incubation .

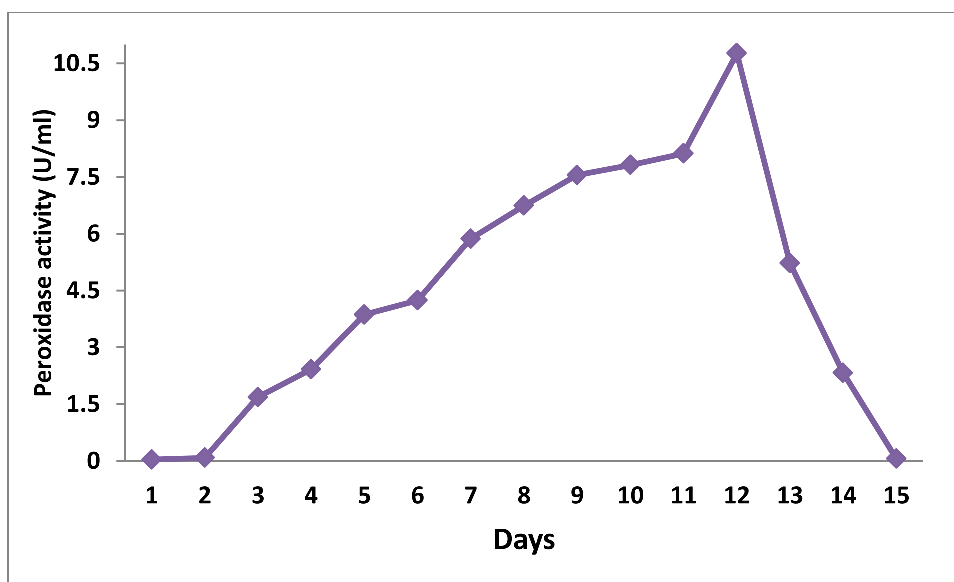


Figure (3) : Effect of incubation period on the production of peroxidase from *A.niger*.

3-3 : Optimum temperature for enzyme production:

The influence of temperature on production of the enzyme showed that peroxidase activity reaching a maximum in 25°C (9.351 U/ml), above this temperature there was a reduction in the peroxidase activity (3.215,0.836 U/ml) in 35, 40 °C respectively (Figure 4). Growth temperature is a very critical parameter which varies from organism to organism and slight changes in growth temperature may affect enzyme production , at higher temperature, due to the production of large amount of metabolic heat, the fermenting substrate temperature shoots up, thereby inhibiting microbial growth and enzyme formation (Bertolin *et al*., 2003)

Similar results were also reported by Gill and Arora (2003) when they shown the highest activity of peroxidase from *Coriolus versicolor*, and *P. chrysosporium*in 25°C , while Ali *et al* (2012) shown the highest activity of lignin peroxidase and manganese peroxidase production by *A. terreus* were 33.6 and 33.1°C, respectively.

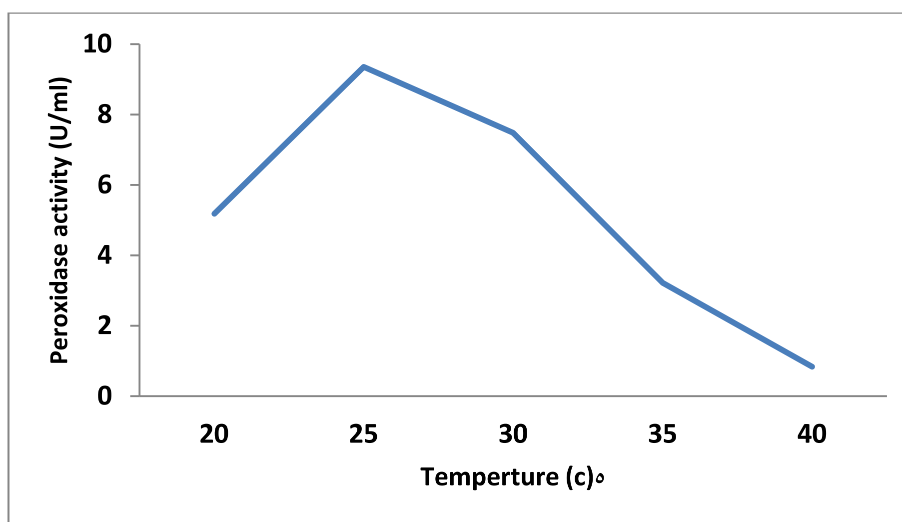


Figure (4) : Effect of incubation temperature on the production of peroxidase from *A.niger*.

3-4 : Optimum pH for enzyme production:

Figure (4) shown the increasing of peroxidase activity with increasing the pH until reach to maximum activity (10.126 U/ml) in pH = 6.5, then it began to decreased in higher pH values (5.972,1.301, 0.073 U/ml) in pH= 7,8, 9 respectively. This study was agreed with Songulashvili *et al* (2007) when they shown the highest activity of peroxidase from *Pleurotus dryinus* and *Phellinus robustus* occurred at the same pH, while Sakurai *et al* (2002) found the highest activity of peroxidase from *Coprinus cinereus* occurred at the pH= 7 .

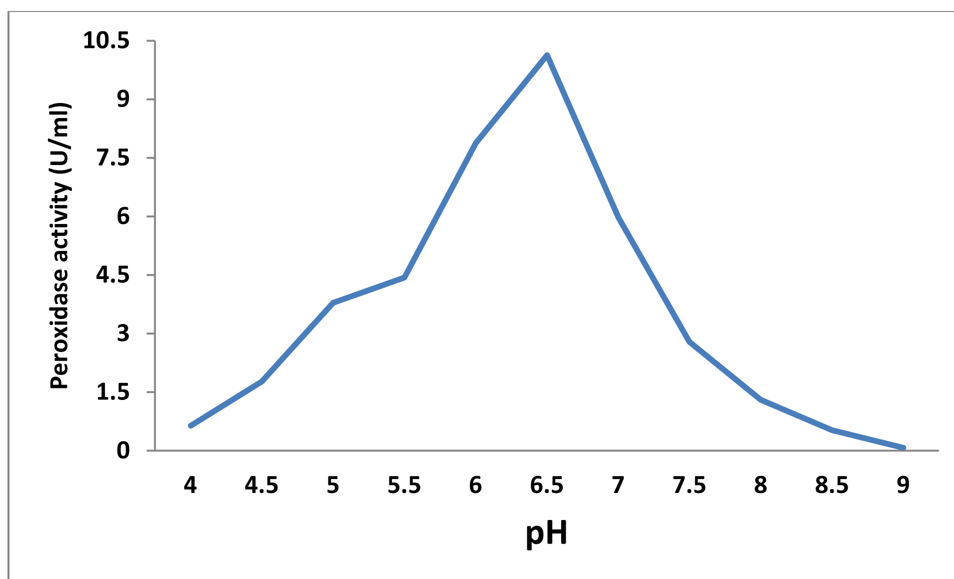


Figure (5) : Effect of pH on the production of peroxidase from *A.niger.*

3-5 : Optimum nitrogen source for peroxidase production:

The over production of enzyme by *Aspergillus niger* was occurred using the peptone as nitrogen source, it gave high titer of peroxidase activity (7.533 U/ml) followed by ammonium nitrate (4.571 U/ml), while the other sources (beef extract, ammonium chloride, and ammonium tartarate) gave low titer of peroxidase activity (3.824, 2.619, 0.631 U/ml) respectively figure (6). Similar results were also reported by Laura *et al* (2010) when they found the highest activity of peroxidase from some white-rot fungi occurred using the peptone as nitrogen source, while the other study like Stajic *et al* (2006) reported the maximum activity of peroxidase from *P. Pulmonarious* using the KNO_3 as nitrogen source.

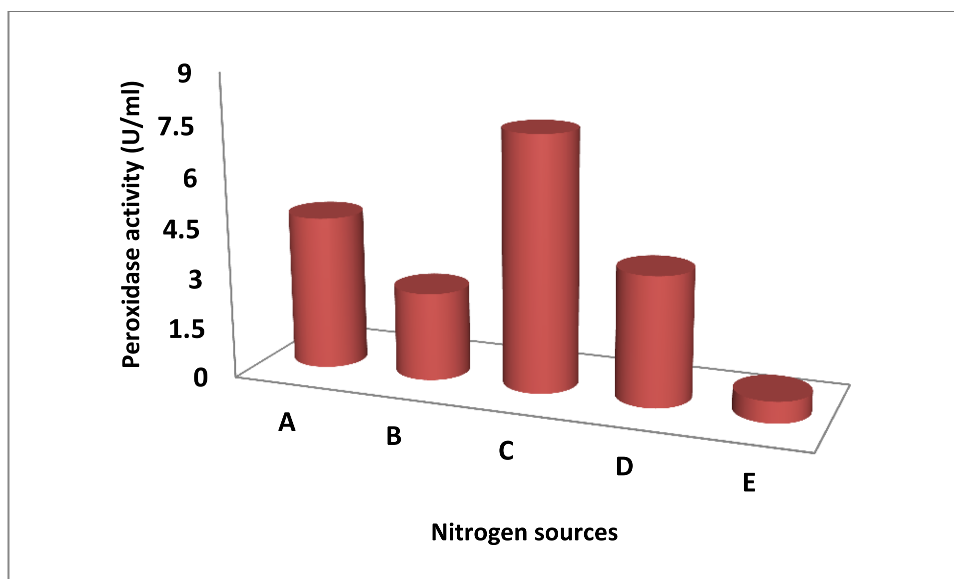


Figure (6) : Effect of nitrogen sources (A: ammonium nitrate, B: ammonium chloride, C: peptone, D: beef extract, E: ammonium tartarate on the production of peroxidase from *A.niger*.

3-6 : Optimum carbon source for peroxidase production:

The figure (7) shown the highest enzyme production by *Aspergillus niger* was occurred using the fructose as the carbon source, it gave high titer of peroxidase activity (8.577U/ml), followed by glucose (5.854 U/ml), while the other sources (sucrose, mannitol, lactose) gave low titer of peroxidase activity (3.751, 2.139, 0.847U/ml) respectively. This study was agreed with Johnsy and Kaviyarasan (2011) when they shown the highest activity of peroxidase from *Lentinus kauffmanii* achieved using the fructose as the carbon source, while Prasher and Chauhan (2013) reported the highest activity of peroxidase from *Grammothele fuligo* achieved using the xylose as the carbon source.

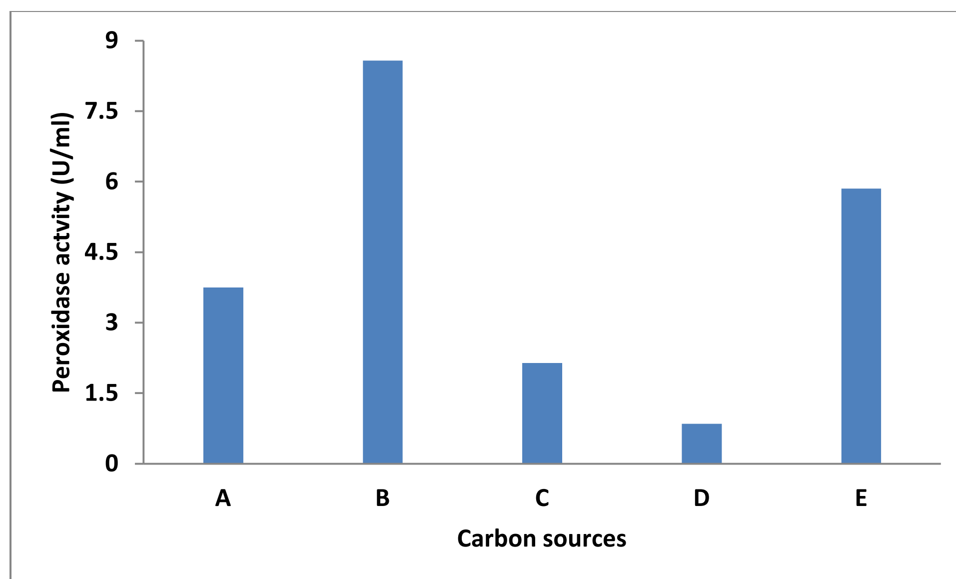


Figure (7) : Effect different Carbon sources (A: Sucrose, B: fructose, C: mannitol, D: lactose, E: glucose) on the production of peroxidase from *A.niger*.

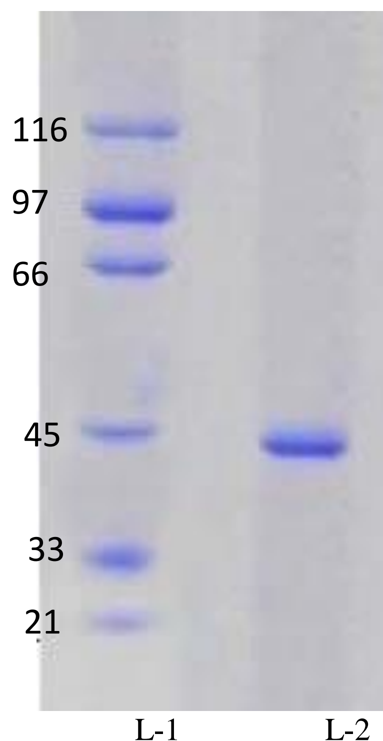
Enzyme purification

The results obtaind for the purification of peroxidase from ***A.niger*** summarized in table **(2).** The workers have also reported that variation in yield and specific activity of purified enzyme from *Bacillus subtilis* (Rao and kavya,2014), *M. Oleifera* (Khatun *etal*. 2012) and soubean (Farzana *etal*.2002).The molecular weight estimated by gel filtration on sephadex G-100 column was approximately 50 kDa while in SDS-PAGE was 40 kDa figure (8).

Table 2. Purification summary of peroxidas produced from *A. niger*

Purification Steps	Enzyme Activity (U/ml)	Protein Content (mg/ml)	Specific Activity (U/mg)	Purification fold
Crude Enzyme	11.6	0.60	19.33	1
$(NH_4)_2SO_4$ Precipitation 70%	11.1	0.40	27.75	1.43
Dialysis	12	0.30	40	2
Ion exchang	14	0.18	77.77	4.02
Sephadex G-100	18	0.12	150	7.75

kDa



Figure(8) SDS-PAGE of purified peroxidase from *A. Niger*. L-1 Protein ladder, L-2 Purified peroxidase by gel filitration

Effect of pH and temperature on peroxidase activity.

Results of enzyme assay showed that the optmium peroxidase activity at pH 7 (Figure 9).The result indicated in figure(10) showed that 35 C^0 was optimum temperature for peroxidase acitivity.The optimum activity of peroxidase purified from *Bacillus subtilis* at pH 6 and optimum temperature at 37 C^0 (Rao and kavya,2014).

The optimum pH and temperature for peroxidase purified from *M.Oleifra* seeds were 6 and 50 C^0 respectively. While the optimum pH for peroxidase activity of *Malva neglecta* was in pH 7 and optimum temperature at 45 C^0(Al-baedri, 2012).

The production of peroxidase from different sample showed higher activity at pH 7 and optimum temperature about 40 C^0 indicating its thermostable nature. These results are very important because these strain produces peroxidase enzyme in an inexpensive source which facilitates its purification. So the produce the peroxidase enzyme in optimum conditions in these study its suitable for industrial application.

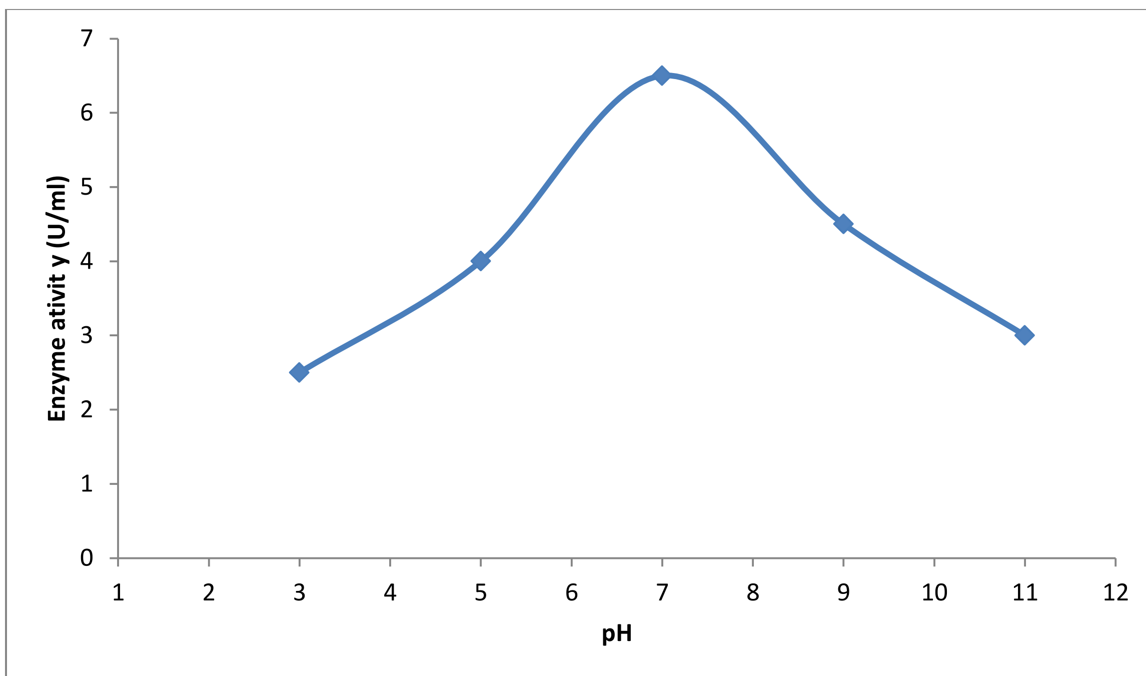


Figure (9): Effect different of pH on peroxidase activity purified from *A. niger*.

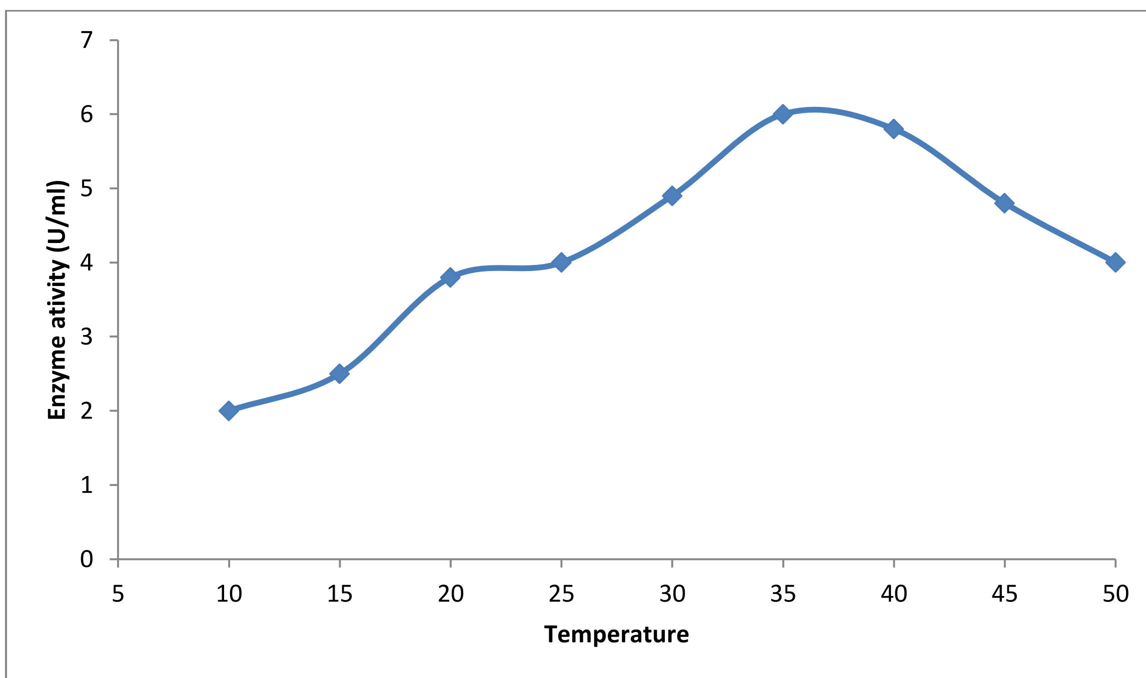


Figure (10): Effect different of temperature on peroxidase activity purified from *A. niger*.

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