# Production of a pharmaceutically important metalloprotease by *bacillus polymyxa* upon biore mediation of some agricultural wastes

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**Abstract:** Agricultural wastes are very rich in nutrients that can be used as complete balanced microbiological media for growing microbes in order to produce valuable commercial products. In this study, a biodegradation process of wheat bran was carried out to produce metalloprotease *enzyme* from *B. polymyxa 1301* strain which is considered to be a hyper producer for that enzyme. Metalloprotease was partially purified by ammonium sulfate precipitation followed by application on Sephadex G-75 column. Gel filtration step resulted in more than 40 times fold purification of the purified enzyme. The enzyme activity was inhibited by EDTA and EDTA disodium (95% and 80%, respectively) at 15 mM concentration. This agro-industrial waste was used as a substrate for economic production of the enzyme from *B. polymyxa* compared with some bacterial isolates collected from different sites in Menofiya governorate, Egypt. Optimum temperature for enzyme activity was 40 °C. It also exhibited a broad pH activity range (4-12) with an optimum pH of 10.
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**Key words:** metalloprotease, B. polymyxa, EDTA and EDTA disodium, agro-industrial waste, Sephadex G-75.

## 1. Introduction

Agricultural wastes all over the world are produced in a huge amount annually (about 140 billion metric tons). Most applicable researches were carried out to use this massive amount of nutrients-rich wastes for production of commercially valuable biomolecules. Protein and enzymes are examples of these biomolecules which are produced via solid state fermentation processes of agro-industrial wastes. Metalloproteases were produced by biodegradation of protein-rich agricultural wastes supplemented with nitrogen sources (Sharma, 2012; Vidali, 2001). Recently, pharmaceutical and biocatalyst enzymes production via different biodegradation processes reached 6.3 percent per year of the world enzyme demand. The world market for enzymes reached $7 billion in 2013. The most demanded industrial enzymes are nucleases, polymerases, lipases, amylases and proteases (Parameswaran *et al*., 2013) Metalloproteases are members of proteases that contain a metal ion at their active site. These enzymes act as catalysts in the hydrolysis of peptide bonds (Hooper, 1996). Zinc metalloprotease is one of the most common members of this class of enzymes, it contains a zinc ion ($Zn^{2+}$) in its active site (Alvarez *et al*., 2006). Other transition metals have been found at the active sites of different metalloproteases such as $Co^{2+}$ and $Mn^{2+}$ (Botelho *et al*., 2011). Generally, metal ions are bound in a nearly tetrahedral conformation at the active site. The metal core is mainly consisted of three amino acid ligands, together with one water molecule which is used for hydrolysis (Dideberg, 1982). There are two major subclasses of metalloproteases: metalloendopeptidases and metalloexopeptidases. Each subclass is named according to the region of hydrolysis in targeted protein at which the reaction takes place. Because metalloproteases play key roles in many normal biological processes, their abnormal activities have been implicated in many diseases such as arthritis, cancer, cardiovascular diseases, nephritis, disorders in the central nervous system, fibrosis, and infection (Hooper, 1994). Recently it has been reported that several zinc dependent proteases have significant roles in the activity of inflammatory cells (Murphy and Lee, 2005). These enzymes play key roles in the responses of cells to their microenvironment. By effecting proteolytic degradation or activation of cell surface and extracellular matrix (ECM) proteins which influence cell differentiation, migration, proliferation and survival (Baker *et al*., 2002). On industrial scale, microorganisms are the most important sources for metalloproteases production. Habitats that contain protein are the best sources to isolate proteolytic microorganisms. Waste products of meat, poultry and fish processing industries can supply a large amount of protein rich material for bioconversion to recoverable products (Gaustevora *et al*., 2005). Many of the organisms produce more than one kind of protease. The type of proteolytic enzyme formed may depend on the composition of the medium. Present study aimed to produce metalloprotease by utilizing agro

industrial waste (wheat bran) as a substrate for bacterial growth followed by purification of the produced enzyme and measuring its activity and stability.

## 2. Materials and Methods

**Bacterial strains**

*B. polymyxa 1301* was used in this study as a metalloprotease-hyper-producing strain. In addition, thirty bacterial isolates previously isolated from Menofia governorate, Egypt were tested for their ability to produce extracellular proteases.

**Media**

Peptone yeast extract (PY) medium contained 10 g peptone, 5 g yeast extract, and 5 g NaCl per liter. PA medium was PY supplemented with 15 g agar agar per liter. The pH value was adjusted to 7.0 by NaOH. PY and PA media were used to activate and to grow the bacterial strains. PA milk medium was PA supplemented with 7.5% skim milk. Physiological saline solution contained 8.5 g NaCl per liter.

Basal medium contained (0.1% $KH_2PO_4$,0.25% NaCl, 0.01%$MgSO_4$.7HO, 0.01%$CaCl_2$).

**Rapid screening for protease-hyper-producing bacteria**

Bacterial isolates were tested for their ability to produce extracellular proteases by growing them on PA milk plates. All plates were incubated at $30^OC$ for 12-18 hours. Colonies that produced considerable clear zones were screened a protease-hyper-producing strains.

**Determination of viable count**

Viable bacterial count (colony forming units, CFU) was carried out as described by (Freshney, R. 1987).

**Monitoring growth and proteolytic activity**

Bacterial cells were activated by growing them overnight on PA plates at $30^OC$. Two ml of PY medium was inoculated with several recently growing colonies and it was allowed to grow for 24 hours at $30^OC$. Hundred ml of PY were inoculated with the above culture and it was allowed to grow at $30^OC$ with shaking to the indicated time. Growth was monitored by measuring the absorbance at 660 nm which was very much correlated with the number of viable cells. At the indicated time 5 ml of the growing culture was taken and centrifuged at 6,000 rpm for 10 minutes. The supernatants were used as a crude enzyme to measure the activity of the metalloprotease.

**Monitoring growth and extracellular protease production during the utilization of wastes**

Bacterial cells were activated by growing on PY medium as described above. A hundred ml basal medium containing 1% wheat bran was inoculated with the above culture. The new culture was allowed to grow at $30^OC$ with shaking at 110 rpm to the indicated time. Samples were centrifuged at 6,000 rpm for 2 minutes and supernatants were used as crude enzyme to measure the protease activity. Growth was monitored throughout the cultivation time by determining the colony forming units (CFU/ml).

**Spectrophotometric method of Protease activity**

The activity of the protease was determined according to the method of Reichard *et al*. (1990) with some modifications. Reaction mixture (2 ml) containing 10 mg casein, 0.9 mM Tris-HCl buffer, pH 7.2, containing 100 µmole of 0.1 mM $CaCl_2$ and 0.1 ml (or an appropriate dilution) of supernatants. The reaction was carried out at $37^OC$ for 30 minutes then it was terminated by the addition of 2 ml 5% w/v trichloroacetic acid (TCA). Reactions were then kept on ice for 30 minutes then samples were centrifuged at 4000 rpm for 10 minutes. The absorbance of the TCA soluble fractions was measured at 280 nm. One unit of enzyme activity was equal to the amount of enzyme that liberates one micromole of tyrosine from casein per 30 minutes at $37^OC$.

**Electrochemical method of Protease activity**

Three separate DNNS-based protamine-sensitive membrane electrodes were used simultaneously to monitor the initial decrease in protamine levels (Chang *et al*., 1999). Experiments were performed by adding 5 l of a 5 mg/ml protamine (Sigma, St. Louis, MO) solution to 1 ml of Tris working buffer (50 mM Tris and 120 mM NaCl, pH 7.4) to yield a final concentration of 25 g/ml protamine. After reaching a steady-state/ non-equilibrium response (3 min), 100 l of a preincubated (5 min) sample mixture containing MP was added to the solution. Sample mixture was composed of centrifuged bacterial culture solution in Tris working buffer. The decrease in the EMF response toward protamine was monitored over a 5-min period by each of the sensors. A calibration plot for MP was constructed by graphing the initial rate of the potential decrease, in mV/min (average response of three sensors), vs MP activity, in IU/ml sample.

**Protein determination**

Determination of soluble proteins was carried out according to Bradford (1976).

**Preparation of agro waste**

The environmental waste (wheat bran) was dried at $60^OC$ for 24 hours and was ground with a laboratory blender. One gram was pretreated by the addition of 10 ml 1 N $H_2SO_4$ and heated at $121^OC$ for 60 minutes. Basal medium II supplemented with 1% of the above treated waste was used to monitor the bacterial growth and the extracellular proteases production. To investigate the effect of acid pretreatment on the growth and the production of protease, untreated samples were used in parallel experiments.

**Purification of the extracellular metalloprotease**

**Precipitation of metalloprotease by $(NH_4)_2SO_4$**

Extracellular enzyme was precipitated by 65% saturation of salt in an ice-bath then it was allowed to stand for 1 hour at $4^{O}C$. The above mixtures were centrifuged at 6,000 rpm for 30 minutes. Pellets were dissolved in a minimal volume of 0.1 M Tris-HCl buffer, pH 7.2 then dialyzed overnight against the same buffer.

**Purification of the alkaline protease by Sephadex G-75 column**

**Fractionation of enzyme sample**

Two ml of the dialyzed alkaline protease was applied to the Sephadex G-75 (1.5 x 20 cm) column. The enzyme was eluted with 0.1 M Tris-HCl, pH 7.5 containing 10 mM $CaCl_2$ at a flow rate of 36 ml/hours. Fractions were collected at $4^{O}C$ after which the absorbance at 280 nm and the enzyme activity were determined. The active fractions were tested separately against certain inhibitors, EDTA and EDTA disodium which are considered to be specific inhibitors of metalloprotease.(Rufo *et al*., 1990) Active fractions were collected and precipitated on ice with solid $(NH_4)_2SO_4$. After standing overnight at $4^{O}C$, the precipitate was removed by centrifugation at 6,000 rpm for 30 minutes at $4^{O}C$. The precipitate was dissolved in a small volume of the above buffer and stored at $-20^{O}C$ to be used in further study.

**Optimum pH for enzyme activity**

Purified extracellular metalloprotease activity was determined over a pH range (4-12). The buffers used in this test were 0.1 M acetate buffer for pH 4-6, 0.1 M Tris-HCl buffer for pH 7-8, and 0.1 M carbonate buffer for pH 9-12.

**Effect of inhibitors**

To study the effect of protease inhibitors on the proteolytic activity, different concentrations EDTA, sodium citrate and sodium oxalate were used by adding the corresponding inhibitor to the reaction mixture, incubated at 37 $^{O}C$ for 1 h. without the substrate fraction and assayed as descried above.

## 3. Results

**Screening for protease-hyper-producing bacteria**

In an attempt to produce extracellular metalloproteases by microorganisms through the process of waste biodegradation, screening was carried out to choose suitable strains for the commercial production of bacterial extracellular enzyme. Thirty bacterial isolates (isolated from different soil samples in Menofiya governorate were screened on PA milk plates to check the ability of the isolates to produce extracellular proteases. The ratio of the whole diameter (diameter of the clear zone including colony size, x) over that of bacterial growth (y) was taken as an indicator for the production of extracellular proteases. (Table 1). Twelve isolates showed relatively high (x/y) values. Results of the screened isolates were compared with *B. polymyxa 1301* which was taken as a metalloprotease hyper-producing strain. *B. polymyxa*was almost three times greater than other isolates.

**Table (1): Screening of different bacterial isolates for their extracellular proteases production**

| Bacterial isolate | Growth diameter (Y cm) | Whole diameter (X cm) | (X/Y) | Production % compared to ref. strain |
|---|---|---|---|---|
| EI001 | 1.4 | 1.5 | 1.07 | 31.2 |
| EI002 | 1.6 | 1.8 | 1.13 | 32.8 |
| EI003 | 1.2 | 1.5 | 1.25 | 36.4 |
| EI004 | 1.5 | 1.7 | 1.13 | 33.0 |
| EI005 | 1.3 | 1.6 | 1.23 | 35.9 |
| EI006 | 1 | 1.1 | 1.10 | 32.1 |
| EI007 | 1.7 | 1.9 | 1.12 | 32.1 |
| EI008 | 1.8 | 1.9 | 1.06 | 32.6 |
| EI009 | 1.8 | 1.9 | 1.06 | 30.8 |
| EI010 | 1.5 | 1.7 | 1.13 | 30.8 |
| EI011 | 1.7 | 2.1 | 1.24 | 36.0 |
| EI012 | 1.4 | 1.7 | 1.21 | 35.4 |
| *B. polymyxa* | 0.7 | 2.4 | 3.43 | 100.0 |

% = The ability of the bacterial isolates to produce proteolytic activity compared to *B. polymyxa 1301*.

**Monitoring enzyme activity during microbial growth curve**

To optimize the whole process of biodegradation, production of extracellular protease was carried out on basal medium that contained different concentrations of treated wheat bran. Enzyme production was compared after growing *B. polymyxa* on the protein rich PY. (Table 2). Production starts early in the exponential phase, to help the microbe in performing all its anabolic processes. Production reached more than 2000 U/ml after three hours of incubation at $30^{o}C$.

Highest enzyme activity was obtained when *B. polymyxa* was grown on 3% treated wheat bran.

Although the enzyme production was almost half the activity obtained when the microbe was grown on PY medium, bioremediation process not only offered a cheap method for enzyme production but also helped in cleaning up an environmental waste.

**Table (2): Extracellular proteases production by *B. polymyxa* during microbial growth**

| Incubation Time (hr) | Enzyme activity after microbial growth on: | | | |
|---|---|---|---|---|
| | 1% wheat bran | 3% wheat bran | 5% wheat bran | PY |
| 1 | 540 | 744 | 587 | 1583 |
| 2 | 597 | 822 | 788 | 2004 |
| 3 | 588 | 854 | 747 | 2141 |
| 5 | 591 | 917 | 770 | 2204 |
| 8 | 602 | 967 | 796 | 2125 |
| 24 | 488 | 1056 | 832 | 1944 |
| 48 | 452 | 903 | 811 | 1913 |
| 72 | 403 | 843 | 701 | 1620 |

**Purification of extracellular metalloprotease produced by B. polymyxa**

*B.polymyxa*cells were grown on PY medium until the proteolytic activity reached its maximum. Cell free supernatant was precipitated with ammonium sulfate (65% saturation) and dialyzed against Tris-HCl. This step resulted in more than 29-foldpurification of the enzyme (Table 3). Dialyzed enzyme was applied to a Sephadex G-75 column at a flow rate of 36 ml/hr. The total proteolytic activity of the enzyme eluted also appeared in a single peak that overlapped with the protein peak. This would indicate that the majority of proteins are actually in the form of proteases. The gel filtration step resulted in more than 64-fold purification.

**Table (3): Purification table of metalloprotease produced by *B.polymyxa*cells**

| Purification step | Mg protein/ml | Metallo protease Units/ml | Total volume (ml) | Total units | Specific activity | Fold purification |
|---|---|---|---|---|---|---|
| Cell free supernatant | 3.1 | 28 | 78 | 2184.0 | 9.0 | 1.0 |
| Pellets after $(NH_4)_2SO_4$ ppt. | 3.4 | 903 | 2 | 1806.0 | 265.6 | 29.5 |
| Pellets after dialysis | 1.9 | 885 | 2 | 1770.0 | 465.8 | 51.8 |
| Gel filtration | 1.1 | 642 | 2 | 1284.0 | 583.6 | 64.8 |

To assure the identity of extracellular protease produced by *B. polymyxa* purified enzyme was assayed in presence of some metalloprotease inhibitors. Inhibitor were added to the reaction mixture in a split sample to compare proteolytic activity. Table (4) shows activity of purified extracellular protease produced by *B.polymyxa*. The table also shows comparison of proteolytic activity after assaying with and without the chelating EDTA compound. The enzyme was totally deactivated with 1 mM EDTA but it was almost intact at different concentrations of sod. oxalate. Residual activity was dropped to almost 60% of its original in the presence of 5 mM sod. citrate. Previous result was compatible with that found in literature. (Griffin and Fogarty, 1973) and itcategorized the extracellular protease produced by the microbe as a metalloprotease.

**Table (4): Effect of some inhibitors on extracellular protease activity**

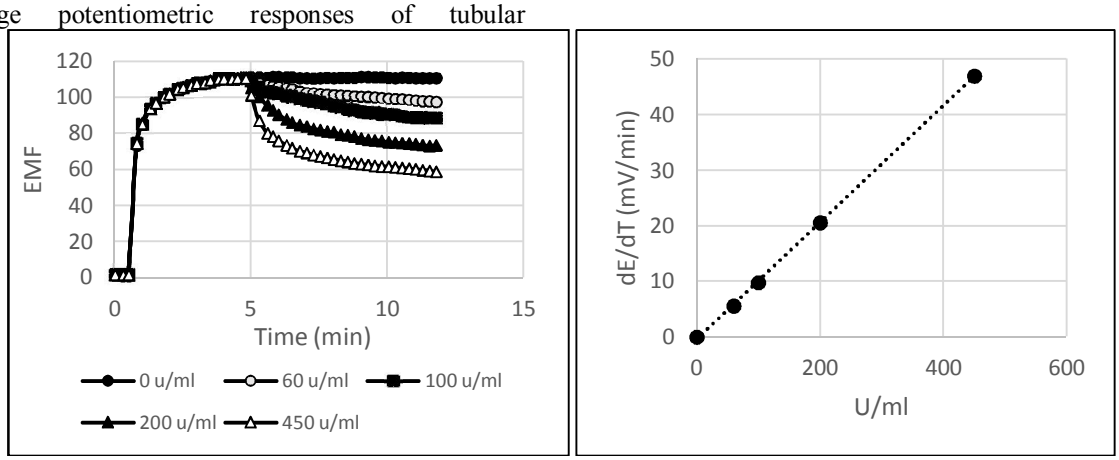
| Inhibitor | Conc. (mM) | Residual activity % |
|---|---|---|
| Without inhibitor | 0 | 100.0 |
| EDTA | 1 | 0 |
| Sodium citrate | 1<br>5 | 84<br>61 |
| Sodium oxalate | 1<br>5 | 98<br>95 |

One unit of enzyme activity was equal to the amount of enzyme that liberates one micromole of tyrosine from casein per 30 minutes at $37^{O}$C.

**Electrochemical enzyme detection**

Protamine-sensitive membrane electrodes have been used to measure trypsin activity (Yun *et al*., 1995). Protamine is an arginine-rich protein which is known to be an excellent substrate to proteases. Polyion-sensitive electrodes have a high response towards protamine (Chang *et al*., 1999).

Metalloprotease activity was detected by measuring the initial rate of decrease in the potentiometric response of the polycation-sensitive membrane electrode towards protamine degradation by the action of enzyme. Figure (1, a) shows the average potentiometric responses of tubular dinonylnaphthalene sulfonate (DNNS)-based protamine-sensitive membrane electrodes toward 25 mg/ml protamine and the effect of adding increased concentrations of metalloprotease. Figure (1, b) shows the average initial rate of potential change (-dE/dt) as a function of metalloprotease activity. All measurements were compared to split samples that were measured with spectrophotometric casein hydrolysis method. Both electrochemical and spectrophotometric enzymatic detection offered a robust and reliable method that can be used to assay for metalloprotease produced by *B.polymyxa.*




(a) (b)

Figure (1). (a) Potentiometric response of (DNNS)-based protamine-sensitive membrane electrodes towards 25 µg/ml protamine and subsequent addition of 100 ml of standard metalloprotease. (b) Average initial rate of potential change (-dE/dt) (for N=3) as a function of metalloprotease activity.

**Optimum pH for enzyme activity**

**Table (5): Optimum pH for activity of extracellular metalloprotease produced by *B. polymyxa***

| pH | Enzyme activity U/ml | Residual activity % |
|---|---|---|
| 4 | 151 | 18.9 |
| 5 | 250 | 31.2 |
| 6 | 764 | 95.4 |
| 7 | 801 | 100.0 |
| 8 | 404 | 50.4 |
| 9 | 314 | 39.2 |
| 10 | 251 | 31.3 |

Results of optimum pH and stability range of extracellular metalloprotease produced by *B. polymyxa*at different buffer systems are shown in table (5). The enzyme retained more than 50% of its activity over the pH range (6-9) with an optimum pH of 7. A very little decrease in the residual activity (less than 5 percent) was shown at pH 6.

To test for the effect of different storage buffers on the residual activity of extracellular metalloprotease produced by *B. polymyxa,* the enzyme was pre-incubated for one hour in different buffer systems (0.1 M acetate buffer for pH 4-6, 0.1 M Tris-HCl buffer for pH 7.0-8.0, 0.1 M carbonate buffer for pH 9.0-10.0 and 0.1 M phosphate buffer for pH 11.0-12.0). Table (6) showed that the residual of the pre-incubated enzyme was slightly affected over the alkaline pH range (8-11). On the other hand, activity was greatly decreased upon incubating the enzyme in acidic pH buffers.

**Table (6): Effect of different storage pH buffer on residual activity of metalloprotease produced by *B. polymyxa***

| pH of storage buffer | Enzyme activity U/ml | Residual activity % |
|---|---|---|
| 4 | 51 | 6.7 |
| 5 | 351 | 46.1 |
| 6 | 503 | 66.0 |
| 7 | 762 | 100.0 |
| 8 | 755 | 99.1 |
| 9 | 695 | 91.2 |
| 10 | 685 | 89.9 |
| 11 | 672 | 88.2 |
| 12 | 661 | 86.7 |

Enzyme was pre-incubated for one hour in different buffer systems (0.1 M acetate buffer for pH 4-6, 0.1 M Tris-HCl buffer for pH 7.0-8.0, 0.1 M carbonate buffer for pH 9.0-10.0, and 0.1 M phosphate buffer for pH 11.0-12.0).

## 4. Discussion

*Bacillus polymyxa*was used together with thirty bacterial isolates, previously isolated from the soil of Menofiya governorate to produce metalloprotease after bioremediation of wheat bran. This agricultural waste offered a suitable and cheap medium for enzyme production from all isolates. Moreover, production was about 3 to 4 fold higher than that of other isolates when *B.polymyxa*was used. The enzyme was purified by $(NH_4)_2SO_4$ precipitation followed by a gel filtration Sephadex G-75 column. Data illustrate that the enzyme was purified to almost 29 fold after the salting out process with a recovery of about 82% of total units. Further purification using the Sephadex G-75 column resulted in increasing the fold purification to about 64 fold with about 59% recovery of total units. After assaying the enzyme with some inhibitors, all the proteolytic activity was attributed to metalloprotease. (Griffin and Fogarty, 1973). Optimum pH of the purified enzyme was about pH 7 with a stability range in the alkaline storage buffers. Produced enzyme was also detected using both spectrophotometric and electrochemical detection methods and both were found to be reliable in enzymatic assay.

Assaying metalloprotease with electrochemical detectors, such as polymer membrane-based ion-selective electrodes offers a number of advantages over spectrophotometric methods especially in cases where samples are highly colored or turbid like bacterial cultures. (Chang *et al*., 1999).

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