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Variation of Protease Production by the Bacteria (*Bacillus fastidiosus*) and the Fungus (*Aspergillus funiculosus*)

Zinnat Shahina[1,*], Mohammad Towhid Hossain[2], Mohammad Abdul Hakim[2]

[1]Department of Microbiology, University of Science and Technology Chittagong, Bangladesh
[2]Department of Microbiology, Chittagong University, Bangladesh

Abstract Protease enzymes perform the hydrolysis of proteins or large polypeptide chains into smaller peptides and amino acids, and thus facilitate their absorption by cells which is produced by different types of microorganisms such as bacteria, fungi, yeast and actinomycetes. Commercially they are very important in different industrial levels and products. The aim of this study was to isolate the protease producing bacteria and fungi and to optimize the media composition that supports the maximum protease production. In the present study the variation of protease production by the bacteria (*Bacillus fastidiosus*, Z_8) and the fungi (*Aspergillus funiculosus*, Z_a) was observed by determining their optimum conditions e.g. incubation period, temperature, pH, substrate specificity and substrate concentration etc, where the results showed that the fungal isolate Z_a under study are good producers of protease than the bacterial isolate Z_8 though it took longer incubation time (5 days) for crude enzyme production in the presence of fructose as carbon source at temperature 35°C with pH 6.0.This might be an indication that protease production can be variable amongst microbes to microbes.

Keywords *Aspergillus Funiculosus, Bacillus Fastidiosus,* Protease, Proteolytic Bacteria, Proteolytic Fungi

1. Introduction

The protease enzymes perform the hydrolysis of large polypeptide chains into smaller peptides and amino acids, and thus facilitate their absorption by cells. Commercially they are very important in different industrial levels and products. Generally these enzymes are isolated from various living sources such as plants, animals, and microbes[1]. A variety of microorganisms such as bacteria, fungi, yeast and actinomycetes are known to produce these enzymes[2]. These proteases have wide applications in leather, laundry, food and waste processing industries[3]. Besides, they are also used in pharmaceuticals, medical diagnostics, lens cleansing, decomposition of gelatin on X- ray films and in textiles as a non-hazardous bioalternative[4-7]. Protease enzymes that are isolated from microbial sources are preferred than the enzymes from plant or animal sources since they possess almost all the characteristics desired for their biotechnological applications[8]. Among the various sources, bacterial proteases are the most significant, compared with animal and fungal proteases. Among bacteria, *Bacillus* species are considered as specific producers of extracellular proteases[9].

But the main draw back in the production of bacterial proteases is the requirement of cost intensive procedures for the separation of enzymes from cells. On the other hand, enzymes from fungal origin offer an advantage of separation of mycelium by simple filtration. Besides these, fungus can be grown in inexpensive substrates[10]. Fungi elaborate a wider variety of enzymes than bacteria do[11]. The application in the leather industry for dehairing and bating of hides to substitute currently used toxic chemicals is a relatively new development and has conferred added biotechnological importance[12].

The activity of an enzyme is due to its catalytic nature. The enzyme activity depends upon the substrate concentration, pH, temperature and other physico-chemical factors[13]. The present investigation is aimed at optimizing the growth conditions of different bacteria and fungi isolated from different sources to enhance the protease production and to compare the amount of protease produced by them.

2. Materials and Methods

2.1. Microorganisms

For the isolation of protease producing microbes different sources such as rotten shrimp and spoiled pulse seeds were used.

2.2. Screening of the Isolates

Primary screening was done by using enrichment technique followed by three methods – Boiled egg albumin degradation, Skimmed milk casein hydrolysis and Gelatin hydrolysis. After primary selection, the organisms were used

* Corresponding author:
zs_mbio@yahoo.com (Zinnat Shahina)
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for secondary screening, where the isolates were examined for the protease activity in liquid medium such as (i) Peptone 2%, yeast extract 1%, dextrose 2%[14], (ii) Tryptone 1%, yeast extract 0.5%, dextrose 0.1%[15], (iii) Gelatin 1%, yeast extract 0.2%, glucose 1%, K_2HPO_4 0.3% KH_2PO_4 0.1%, $MgSO_4.7H_2O$ trace[16] were used.

2.3. Measurement of Enzyme Activity

Protease activity of selected isolates was done by using the modified method of Hayashi et al[17], as followed by Meyers and Ahearn[18]. In this method crude enzyme solution (3 ml) was incubated with 3ml of citrate phosphate buffer and 3ml of 1% casein at 35°C in a water bath. The reaction was stopped by adding 5ml of 20% TCA. After one hour, the solution was filtered by Whatman no. 40 filter paper (Ashless). One ml enzyme substrate mixture was taken into a test tube and 2ml of 20 % Na_2C0_3 and 1 ml of Folin Ciocalteu Reagent was added and shaken well and waited for 30 minutes. After this fixed time period 6 ml distilled water was added to it and absorbance of the solution was measured at 650 nm in a spectrophotometer. Enzyme activity was calculated by measuring mg of tyrosine equivalent released and compared with the standard. One unit (U) of enzyme activity represents the amount of enzyme required to liberate 1 µg of tyrosine/ml under standard assay conditions.

2.4. Biomass Yield

For fungal isolate the biomass residue was dried at 70°C to a constant weight and the amount of biomass was calculated by subtracting the weight of filter paper as the supernatant was collected by what man no. 1 filter paper. The yield was expressed as mg g^{-1} of protein. On the other hand bacterial biomass was determined by measuring the absorbance at 600 nm[19].

2.5. Optimization of Culture Conditions

2.5.1. Effect of Incubation Time, Temperature and Medium pH

In the present study the effect of culture conditions were observed at different incubation time (1, 2, 3, 4, 5, 6 and 7 days), pH (5.0, 6.0, 7.0, and 8.5) and temperature (10°C, 27°C, 37°C and 45°C) and their biomass characteristics, biomass yield and protease productions were recorded.

2.5.2. Effect of Carbon and Nitrogen Sources

For the production of extracellular proteases different carbon and nitrogen sources were studied in the liquid medium[15]. Sucrose, dextrose, fructose, starch or maltose were used as carbon source and peptone was used as nitrogen source to the medium and their effect on the production of protease, extracellular protein and biomass yield were recorded.

2.6. Optimum Conditions for Crude Enzyme Activity

To determine the optimum conditions for the crude enzyme activity several factors such as pH (5.0 to 8.5), temperature (35 to 45°C), incubation time (50, 60, 70, 80 and 90 minutes), Substrate concentration (i.e. 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%) and substrate specificity were observed.

3. Result and Discussion

By using enrichment techniques five fungal isolates (ID as Z_a, Z_b, Z_c, Z_d and Z_e) and eleven bacterial isolates (ID as Z_1 to Z_{11}) were isolated from spoiled pulse and dry rotten shrimp respectively.

3.1. Screening and Identification of Selected Isolates

All of these isolates were primarily screened by protein hydrolysis method and found that two fungal strains (Z_a and Z_c) and two bacterial strains (Z_8 and Z_{10}) were vigorously hydrolyzed the egg albumin, skimmed milk casein and gelatin. Among them the fungal isolate Z_a and the bacterial isolate Z_8 were the potent protease producers in the liquid medium that were finally selected for protease production. The selected fungal isolate was tested on the basis of their colony color, spore size, shape, arrangement, different types of conidiophores and sporangiophores, presence or absence of other special morphological features etc and compared with the standard description of "A manual of soil fungi"(Table.1)[20]. The fungal isolate was provisionally identified as Aspergillus funiculosus G. Smith (Fig.1.a, b)

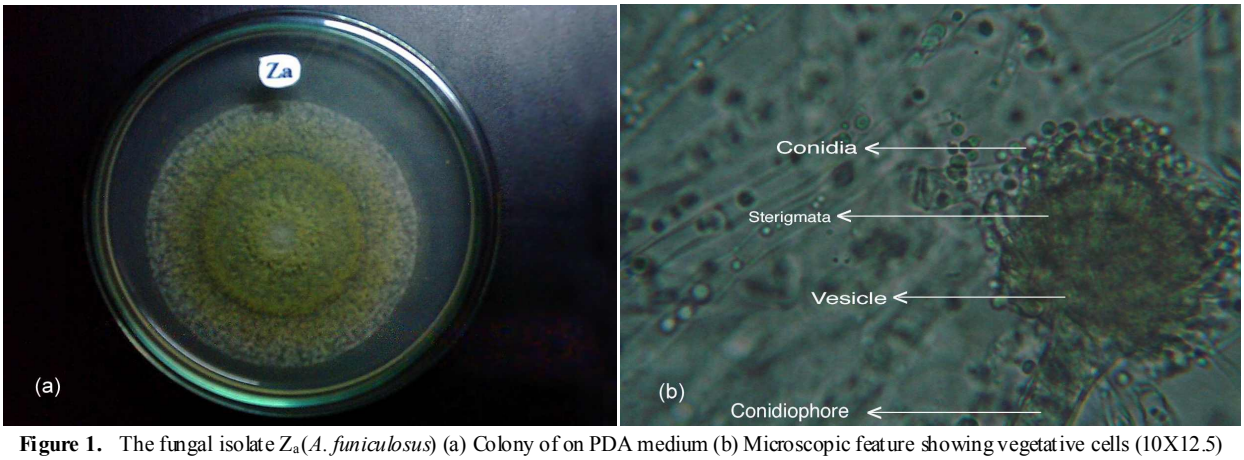


Figure 1. The fungal isolate Z_a(*A. funiculosus*) (a) Colony of on PDA medium (b) Microscopic feature showing vegetative cells (10X12.5)

Table 1. Morphological and cultural characteristics of the fungal isolate Z_a

Sample	Pulse
Vegetative mycelium	Mycelium long, septate , branched, hyaline, Colonies rapidly grown with abundant substratum mycelium
Foot Cell	Present
Conidiophores	Conidiophores mostly rise directly from the substratum, smooth walled conidiophores are long, non septate, 172.9 to 332.5 µm in length and >6µm in breadth.
Conidial head	Conidial heads globose and about 22.61 to 32.5 µm in diameter,fertile whole head and shade of green color.
Sterigmata (Phialids)	9.7 µm in length, uniseriate
Conidia	Conidia smooth walled and light green colors round shaped, 3.23 µm in diameter.
Colony characters in PDA plate	Olive green colonies having white spreading mycelium.
Colony characters in Czapeks medium	Growth slow in Czapeks medium, whitish mycelium whitish mycelium and very scanty growth, green colors spores no substratum color.
Growth in PDA slant	After 24 hours, whitish mycelium found on PDA slant.
Substrate color	The colony change the color of the substratum (PDA) from colorless to purple.
Identification	Above characteristics indicate that the isolate Z_a belongs to the genus ***Aspergillus*** and closely related to the species ***A. funiculosus,*** G.Smith

Table 2. Morphological, cultural, physiological and biochemical characteristics of the bacterial isolate Z_8

Sample	Shrimp									
Vegetative cells	Short rod, arranged in single, pair, short to long chain, length 4 to > 5 µm, width 1 to > 2 µm									
Spore staining	Spore former, shape- round, position-central									
Gram staining	Gram positive									
Acid fast staining	Non acid fast									
Motility test	Positive									
Agar colonies	Form- Rhizoidal, Elevation- Raised, Margin -irregular, Color- white color colony.									
Agar slant	Arborescent									
Nutrient broth	Turbid sedimentary growth.									
Glucose broth	Turbid sedimentary growth.									
Asparagine broth	Negative									
Potato slant	Filiform									
Catalse test	Positive									
Deep glucose agar	Surface growth and growth throughout the medium									
Oxygen relationship	Aerobic and facultative anaerobic									
Growth in synthetic medium	Negative									
Inorganic salt medium	Negative									
Growth in citrate medium	Turbid growth with sedimentation									
Liquefaction of gelatin	Positive									
Proteolysis test	Coagulated egg albumin degraded.									
Casein hydrolysis	Hydrolyzed the skimmed milk casein									
Gelatin hydrolysis	Positive.									
Indole test	Negative									
Nitrate reduction	Positive									
H_2S Production	Negative									
Methyl red test	Negative									
Voges- Proskaur test	Negative									
Starch agar	Negative									
Growth at different temperature (ºC)	10 -	22 +++	2 +++	37 +++	45 +	50 -				
Growth at different NaCl concentration (%)	0 +++	1 +++	2 +++	3 +++	4 +++	5 ++	6 ++	7 +	8 +	9 -
Urease test	Negative									
Oxidase test	Negative									
Fermentation of different carbohydrates	Pentoses-acid and gas not produce from Arabinose, Rhamnose, Xylose. Hexose's-acid and gas not produce from Glucose , Fructose,Galactose. Disaccharides - acid and gas not produce from Sucrose, Lactose. Polysaccharides-acid and gas not produce from Raffinose. Sugar alcohol-acid and gas not produce from Manitol, Glycerol.									
Identification	From the above characteristics the isolates Z_8 is found close to the genus ***Bacillus*** and provisionally identified as ***B. fastitdiosus*** den Dooren de Jong 1929, but the isolates was found to differ in Nitrate reduction.									

On the other hand bacterial isolate was characterized on the basis of morphological (including size, shape; arrangement of the cell; presence and absence of spores; acid fast reaction, gram reaction), cultural, physiological and biochemical properties. Then the isolate was compared with the standard description of "Bergey's Manual of Determinative Bacteriology, 8th edition" (Table.2)[21]. The bacterial isolate Z_8 was found to belong to the genus *Bacillus* and provisionally identified as *B. fastidiosus* (Fig. 2.a,b)

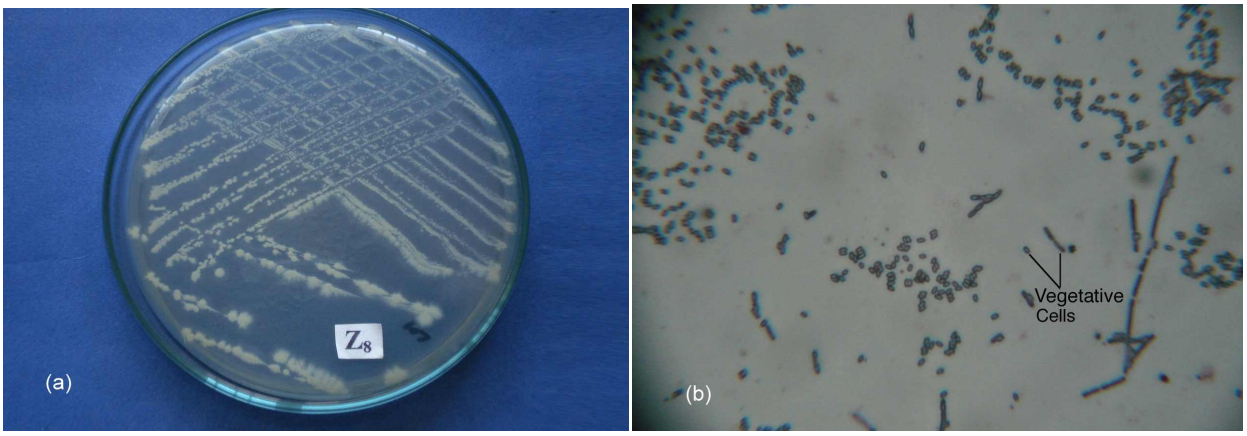


Figure 2. The bacterial isolate Z_8 (*B. fastidiosus)* (a) Colony on NA medium (b) Microscopic feature showing vegetative cells (40 X 12.5)

3.2. Optimization of Different Cultural Conditions

The present investigation was aimed to optimization of medium components which have been predicted to play a significant role in enhancing the production of proteases[22]. So, proper combination of various cultural conditions can be established for *A. funiculosus* and *B. fastidiosus* in order to suit for high secretion of protease.

3.2.1. Effects of Incubation Time

In the present study the isolate *A. funiculosus* showed maximum activity (85.4 U/ml) after 5 days of incubation period which was similar with other research report[23]. On the other hand the bacterial isolate showed maximum protease activity (29.16 U/ml) after 2 days of incubation (Table.3).

Table 3. Effects of incubation period on the production of protease by the isolate Z_a (*A. funiculosus)* and Z_8 (*B.s fastidiosus*)

Incubation Period (in days)	Incubation Period (in hours)	Color and pH after incubation				Biomass characteristics		Biomass yield (absorbance at 600 nm)		Protease activity (U/ml)	
		Color		pH							
Z_a	Z_8	Z_a	Z_8	Z_a	Z_8	Z_a	Z_8	Z_a	Z_8	Z_a	Z_8
3	24	Red oxide	Red oxide	5.60	5.17	Surface growth with scanty sporulation	Sedimentary growth	380	0.960	34.86	19.72
4	48	Red oxide	Red oxide	5.73	5.20	Surface growth with light sporulation	Turbid growth with sedimentation	490	1.009	48.61	29.16*
5	72	Tobacco brown	Red oxide	6.08	5.55	Surface growth with whitish mycelium	Turbid growth with sedimentation	520*	1.021*	85.4*	27.7
6	96	Sand gold	Red oxide	6.10	5.66	Surface growth with light green sporulation	Turbid growth with sedimentation	340	0.957	73.61	25.4

Notes: Incubation temperature: 27 for Z_a and 37ºC for Z_8 and Medium pH: 6, Enzyme substrate reaction pH and temperature: 7.0 and 40ºC, * Maximum biomass yield/ enzyme activity

3.2.2. Effects of pH

Both of the isolates were allowed to grow in media of different pH ranging from 5.0 to 9.0. Maximum enzyme production was recorded at pH 5.0 and 7.0 by the isolates Z_a and Z_c respectively (Table.4).

Table 4. Effect of medium pH on the production of protease by the isolate Z_a (*A. funiculosus)* and Z_8 (*B. fastidiosus*)

Medium pH	Color and pH after incubation				Biomass Characteristics		Biomass yield (mg/gm of substrate)		Protease activity (U/ml)	
	Color		pH							
	Z_a	Z_8	Z_a	Z_8	Z_a	Z_8	Z_a	Z_8	Z_a	Z_8
5	Red oxide	Golden brown	6.57	5.83	Surface growth with whitish mycelium	Turbid growth	450	0.613	141.7*	20.14
6	Red oxide	Tobacco brown	6.90	5.85	Surface growth with whitish mycelium	Turbid growth	600*	0.944	106.25	23.61
7	Red oxide	Red oxide	7.17	6.71	Surface greenish sporulation	Turbid growth with sedimentation	510	1.007*	89.5	48.61*
8.5	Red oxide	Red oxide	7.14	6.71	Surface greenish sporulation	Turbid growth	450	0.982	34.72	25

Notes: Incubations period: 5 days for Z_a and 48 hours for Z_8 ,Incubation temperature: 27 and 37ºC respectively, Enzyme substrate reaction pH and temperature: 7.0 and 40ºC, * Maximum biomass yield/ enzyme activity

3.2.3. Effects of Temperature

Enzyme activity recorded at different temperatures revealed that the *A. funiculosus* yielded maximum protease production at 27ºC and *B. fastidiosus* at 37ºC (Table.5). Related studies also reported that protease production by *Bacillus* sp was best at 37°C[24,25]

Table 5. Effects of temperature on the production of protease by the isolate Z_a (*A. funiculosus)* and Z_8 (*B. fastidiosus*)

Incubation temperature (ºC)	Color and pH after incubation				Biomass characteristics		Biomass yield (absorbance at 600 nm)		Protease activity (U/ml)	
	Color		pH							
	Z_a	Z_8	Z_a	Z_8	Z_a	Z_8	Z_a	Z_8	Z_a	Z_8
10	Red oxide	Red oxide	6	7	No growth	No growth	00	00	00	00
27	Copper leaf	Golden brown	6.71	5.70	Surface growth with whitish mycelium	Turbid growth with sedimentation	450*	1.802*	109.02*	34.72
37	Sand gold	Golden brown	7.54	5.63	Surface growth with whitish mycelium	Turbid growth with sedimentation	390	1.298	60.42	55.5*
45	Red oxide	Red oxide	6	5.80	No growth	Turbid growth	00	0.465	00	13.9

Notes: Incubations period: 5 days for Z_a and 48 hours for Z_8 ,Incubation pH: 7, Enzyme substrate reaction pH and temperature: 7.0 and 40ºC, * Maximum biomass yield/ enzyme activity

3.2.4. Effects of Carbon and Nitrogen Sources

Table 6. Effects of carbon and nitrogen sources on the production of protease by the isolate Z_a (*A. funiculosus)* and Z_8 (*B. fastidiosus*)

Nitrogen source	Carbon source	Color and pH after incubation				Biomass Characteristics		Biomass yield (mg/gm of substrate)		Protease activity (U/ml)	
		Color		pH							
		Z_a	Z_8	Z_a	Z_8	Z_a	Z_8	Z_a	Z_8	Z_a	Z_8
Peptone	Dextrose	Golden brown	Red oxide	6.57	6.71	Surface greenish sporulation	Turbid and sedimentary growth	490	1.002*	137.06	47.86*
Peptone	Sucrose	Golden brown	Red oxide	6.43	6.69	Surface whitish mycelium	Turbid and sedimentary growth	450	0.810	145.5	35.05
Peptone	Maltose	Red oxide	Pale mandarin	6.37	6.05	Surface greenish sporulation	Turbid growth	380	0.949	117.8	27.25
Peptone	Fructose	Golden brown	Golden yellow	6.45	6.49	Surface greenish sporulation	Some sedimentation	496*	0.978	214.7*	39.09
Peptone	Starch	Golden brown	Golden yellow	6.86	6.23	Surface greenish sporulation	Turbid growth	360	0.725	200.1	31.06

Notes: Incubations period: 5 days for Z_a and 48 hours for Z_8 ,Incubation temperature: 27 and 37ºC and Medium pH: 5 and 7respectively, Enzyme substrate reaction pH and temperature: 7.0 and 40ºC, * Maximum biomass yield/ enzyme activity

Various carbon sources such as sucrose, fructose, maltose, starch and dextrose were used to obtain maximum protease production. When peptone was present as nitrogen source in growth media in that case fructose and dextrose increased the protease production compared to other carbon source for *A. funiculosus* and *B.fastidiosus* respectively (Table.6). Many authors reported variability of carbon and nitrogen sources with different organisms[26-32].

3.3. Factors Involved in Enzyme Activity

Different factors such as pH, temperature, incubation time, substrate concentration play an important role for highest protease activity. So determination of these factors is necessary which help us to find out different limiting factor for maximum activity of proteases from microbial sources.

3.3.1. Effect of Reaction Time

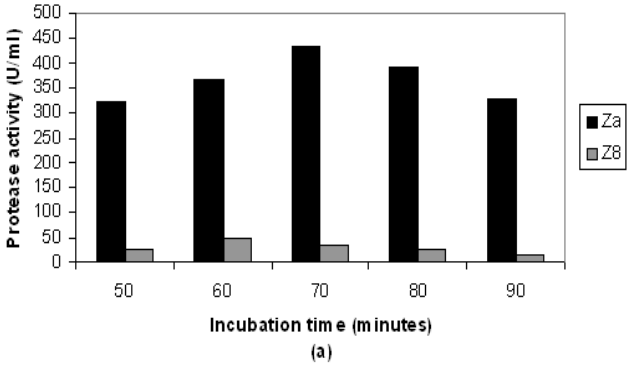
At 40ºC the crude enzyme of fungal and bacterial isolates showed highest protease activity when the reaction mixture were allowed to react for 70 and 60 minutes respectively (Fig. 4 a).

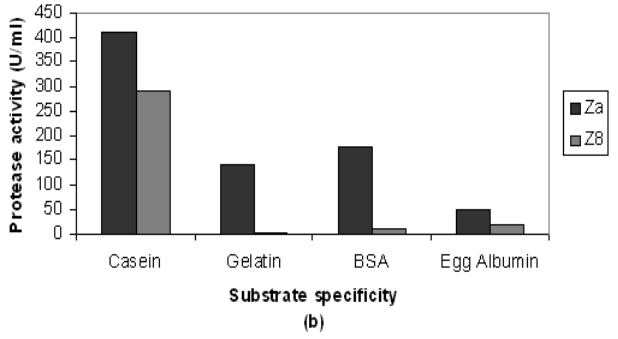
3.3.2. Substrate Specificity

To observe the efficiency of protease activity during enzyme-substrate reaction different substrate such as casein, gelatin, bovine serum albumin (BSA) and egg albumin were used. Among this casein gave the highest value of activity with the crude of fungal and bacterial isolate (Fig.4 b).

3.3.3. Substrate Concentration

Activity of protease has been shown to increase when the reaction mixture were allowed to react with different substrate concentration (0.5 to 3.0%). During enzyme-substrate reaction maximum protease activity was found in 1.5 % and 2.5 % casein for fungal and bacterial isolate respectively (Fig.4 c).





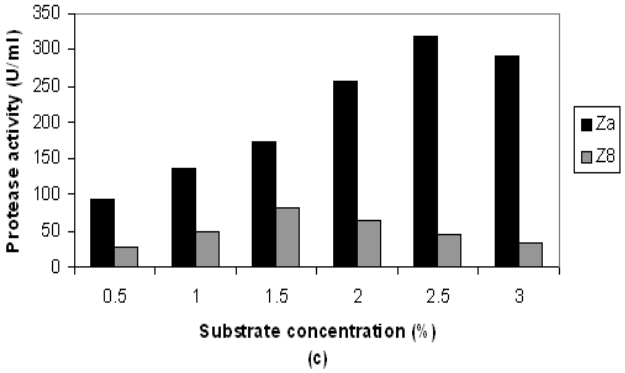
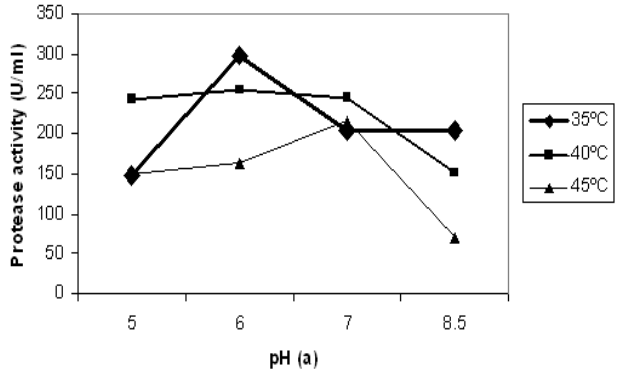


Figure 4. Effects of various factors on the activity of protease of crude extract of fungal and bacterial isolate (a) Incubation time (b) Substrate specificity and (c) Substrate concentration

3.3.4. Effect of Temperature and pH

Protease activity increased with increase in temperature ranged from 35ºC to 45ºC. Maximum activity of protease (76.38 U/ml) was obtained at 45ºC with pH 7.0 by *B. fastidiosus* (Fig.5a).

On the other hand*A. funiculosus* showed highest enzyme activity (298 U/ml) at 35ºC with the reaction pH 6.0 (Fig.5b). Similar results at acid to neutral pH and different temperature was also reported by many authors[23,25, 27,33,34].



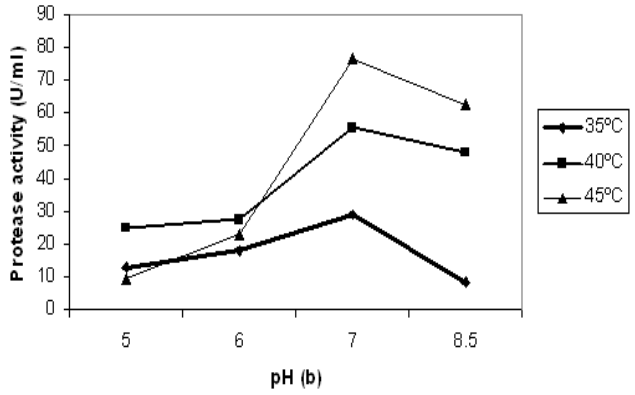


Figure 5. Effects of temperature and pH on the activity of protease of crude extract of (a) fungal isolate and (b) bacterial isolate

4. Conclusions

The aim of this research work was to isolate and identify high protease producer from different sources. This might be an indication that the fungal strain *A. funiculosus* would produce sufficient amount of protease which could find applications in industry and biotechnology. Further work with the split of many other factor and interactions of each factors may provide clear picture about maximum production of protease by the selected isolates. In the present study all of the figures showed that the fungal isolate produced an increase amount of protease enzyme than the bacterial isolate and in case of both isolates it also revealed that protease activity was increased by specific factors. Further investigation on these two species can possibly reveal their potentiality as a source of protease for any biotechnological approach.

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