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Production, Purification and Applications of Thermostable Slaughterhouse (SH), Fish (FW) and Poultry (PW) Wastes Protease(s) Under Solid State Fermentation (SSF) Conditions.

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Abstract

Proteases constitute one of the most important groups of industrial enzymes, accounting for about 60% of the total enzyme market. Proteases due to their huge application spectrum in various biotechnological processes have been the focus of intense research for many decades. However, most of the available proteases lack desired properties from industrial application view point; therefore, search for better and efficient thermostable alkaline proteases, is a continuous practice.In the current study, five thermophilic bacterial strainsviz*B. brevis* I-4, *B. brevis var. gelatinoamylolyticus* I-2, *B. brevis var. gelatinoxyloureamylolyticus* I-5,*B.brevisureamylolyticus* I-7 and *B.brevisxyloamylolyticus* I-8were screened for thermostable protease(s) production using three different types of wastes as substrates viz. slaughter house wastes (SHW), fish wastes (FW) and poultry wastes(PW). Among these bacterial isolates, the most potent species viz. *B. brevis*, I-4, *B. brevis* var. *gelatinoamyloliticus*,I-2 were chosen for further investigations for three proteases enzymes production.The optimal conditions for three enzymes were temperature, 55°C;substrate concentration (gelatin g/l),2; incubation period,24h.; pH,7; inoculum volume,1 ml; flask volume,250ml; incubation conditions, static; carbon source, starch; nitrogen source; sodium nitrate for DSHW protease and potassium nitrate for both FW and PW proteases; amino acid, glycine for DSHW protease and L-Proline for both FW and PW protease; mineral salts, sodium nitate and potassium di-hydrogen phosphate for DSHW protease and potassium di-hydrogen phosphate and magnesium sulphate for both FW and PW protease and finally metal ions (activators and inhibitors), sodium selenite for all produced three proteases under investigation. The produced thermostable enzymes under all optimal conditions were purified by Sephadex G200 and G100. With purification folds, 11 and 16.83; 8 and 14.45; 7, 41 and 14.16 for DSHW; FW and PW proteases respectively. The optimal parameters for three purified proteases enzymes were characterized as flowing: enzyme concentration, 0.125ml %; substrate concentration (gelatin); 0.1 %; incubation temperature, 55°C; pH,7; incubation period, 60 hours for three proteases enzymes. The results emphasized the possibility of the production of thermostable proteases for the application under industrial scale from SHW, FW and PW that could be produced per tons annually. Moreover, the application of the thermostable proteases on leather and the purified proteases as bio-detergent gave promising results to their use in such industries on a large scale.

Keywords: Thermophilic bacteria; Thermostable Proteases Enzymes; Production; Purification; Solid State Fermentation (SSF).

Introduction

Proteases are essential constituents of all forms of life on earth including prokaryotes, fungi, plants and animals. Proteases are highly exploited enzymes in food, leather, detergent, pharmaceutical, diagnostics, waste management and silver recovery (11; 49; 84;100;101;68).Proteases are commonly classified according to their optimum pH as acidic protease, neutral protease and alkaline protease. There have been extensive researches on functions of acidic and alkaline proteases. They constitute59% of the global market of industrial enzymes, which is expected to exceed $ 2.9 Billion by 2012 (26). Proteases have been isolated and characterized from several bacterial and fungal spp. Bacterial proteases are the most significant, compared with animal and fungal proteases (103).Enzymes from *Bacillus* spp. are known for poly-extremotolerance, capable of functioning in adverse ecological conditions. Protease production from several *Bacillus* spp. has been reported. In biotechnological processes for protease production, *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. cereus* have become most popular due to their excellent fermentation properties, high product yields and the complete lack of toxic by-products. In addition, the vast diversity of proteases in contrast to the specificity of their action has attracted worldwide attention in attempt to exploit their physiological and biotechnological applications in various industries (76; 77; 51; 90).

Considering polluting chemical based manufacturing/production technologies, there is immense emphasis on development of enzyme based environmentally-friendly/green technologies. However, enzymes being biological molecules are quite fragile for hostile industrial processes; therefore, there is quest for robust enzymes capable of functioning well under industrial conditions (88; 89; 51).

Microbial proteases represent the largest group of enzyme of commercial importance. Proteases are hydrolytic enzymes, which find applications in wide range of industrial sectors viz. food, feed, pharmaceutical, leather, detergent, textile and several others (32). Proteases constitute one of the major groups of industrial enzymes and accounts for 60% of the total enzyme sales with two thirds of them produced commercially from microbial origin (86; 21). Despite huge application potential, utilization spectrum of proteases is limited due to lack of industrially desirable characteristics among available proteases. Considering that most of the industrial processes are accomplished under hostile conditions including extremes of temperatures and pH, presence of inhibitors etc., proteases intended for industrial application must have robustness against solvents, surfactants, oxidants and stability at high temperature and pH (87). Furthermore, such broad range thermostable, pH-stable, organic solvent resistant proteases may find novel applications in pharma, diagnostic, detergent, tannery, effluent treatment, and other industries. Hence, attention is focused currently on the finding new protease producing microorganisms so as to meet the requirements of industry (88; 89).

Solid-state fermentation (SSF) defined as those in which microbial growth and product formation occur at or near the surfaces of solid materials (61; 81). Solid –state fermentation (SSF) offers potential advantages over submerged –liquid fermentation (SLF), such as superior enzyme productivity, process simplicity, lower capital investment and energy requirement, lower waste output and ease in product recovery. Solid–state fermentation are widely used in the manufacture of some products. Among the microbial products in international and national markets, enzymes occupy a prominent place and large monetary turnover. A number of enzymes has been economically manufactured in Japan and oriental countries by using solid-state fermentation, though submerged fermentation is relied on in European and Western countries. This was mainly due to the due to the neglect of solid-state fermentation in these latter countries. Enzyme production in solid-state fermentation includes amylases, glucoamylases, cellulases, pectinases, lipases, proteases and xylanases (101; 81).

The slaughterhouses wastes are important pollutants factors for the environment. Many pathogenic microorganisms can grow on them; these may cause many diseases for man and animals. These microorganisms secrete number of enzymes, which play important role in the pathogenesis of bacterial infections. These enzymes facilitate the penetration of destruction of host tissues as exemplified by clostridia and certain other bacterial infections. In gas gangrene caused by species of clostridia, enzymes such as collagenases, hyaluronidases and protease aid the organism in destroying tissues.

Fish offal, a major fishery byproduct, is another food processing waste. The disposal of which causes environmental problems. Direct use of fish waste as organic fertilizer id hampered by its foul smell, a problem that is not totally solved by the composting of fish offal. A new approach to fish water composting has been developed, in which fisheries wastes are mixed with sphagnum peat and composted. It is claimed that nearly all the NH_3 produced during the composting is absorbed as NH_4 by the moist acidic peat and that the final product meets, or exceeds, all criteria set for a high quality product, including earthy odor and appropriate concentrations of organic and inorganic nutrient. Fish offal contains high nitrogen content. As a result, fish offal hydrolysate was used as supplemented nitrogen source for production of beneficial fungi on apple processing wastes under solid-state fermentation. Feathers, which are almost pure bacteria keratin protein (90% or more),are produced in large amounts as a waste byproduct as poultry processing plants. Millions of tons of feathers are produced annually worldwide and represent a potential alternative to more expensive dietary ingredients for animal feed. However, the current processes to obtain feather meal are expensive and destroy certain amino acids yielding a product with poor digestibility and variable nutrient quality.

Considering the importance of robust thermostable microbial proteases with process suitability for industrial applications, the current study reports a thermoalkaline protease producing bacteria viz. *B.brevis*, I-4 and *B.brevis var. gelatinoamylolyticus*, I-2 and optimization of cultural conditions for enhanced protease production. Three produced enzymes produced under all optimal conditions were purified and applied in leather and laundry detergent industry.

Materials and Methods

I-Media used:

1- **Growth medium:** Nutrient agar medium (85): It consists of (g/L): Peptone , 5; NaCl,5; Beef extract, 3. and Agar ,15 and distilled water up to 1000ml. Dissolve by heating , adjust pH at 7 and sterilize at 121 °C for 15 min.

2- The Production media:

a- The mineral salts of Dox's medium (MDM) (25) supplemented with slaughter house wastes (SHW) and hence (DSHW) medium: It consists of (g/L) slaughter house wastes, 100; $NaNO_3$,2; KH_2PO_4,1; $MgSO_4.7H_2O$,5;KCL,5;$FeSO_4$,0.001 and distilled water up to 1000 ml. Dissolve by heating , adjust pH and 7 and sterilize at 121°C for 15 min.

b- The mineral salts of Dox,s medium (MDM) supplemented with Fish wastes (FW) and hence called (DFW) medium: It consists of (g/L) fish wastes ,100; $NaNO_3$,2; KH_2PO_4,1; $MgSO_4.7H_2O$,0.5; KCl,0.5; $FeSO_4$,0,001 and distilled water up to 1000 ml. Dissolve by heating , adjust pH at 7 and sterilized at 121°C for 15 min.

c- Mineral salts of Dox.s medium (MDM) supplemented with poultry wastes (PW) and hence called (DPW) medium: It consists of (g/L) poultry wastes, 100; $NaNO_3$,2; KH_2PO_4,1; $MgSO_4.7H_2O$,0.5; KCl ,0.5; $FeSO_4$,0.001 and distilled water up to 1000 ml. Dissolve by heating , adjust pH at 7 and sterilize at 121°C for 15 min.

3-Protease assay medium:

This was carried out according to gelatin clearing zone (GCZ) technique (7). The assay medium consists of (g/L): Gelatin, 10; Agar, 20; (0.2M) phosphate buffer up to 1000ml.

II- Bacteria used: Five different thermophilic bacterial strains(isolated from extreme environment) viz. *B. brevis*,I-4 ; *B. brevis* var. *gelatinoamylolyticus* , I-2; *B. brevis* var. *gelatinoxyloureamylolyticus*,I-5; *B. brevis ureamylolyticus*, I-7 and *B. brevisxyloamylolyticus*, I-8 were isolated from the fresh water hyacinth collected from Auseem area , one of the most famous districts belonging to El- Giza Governorate , Egypt, isolated and identified by Omar(69), Botany & Microbiology Dept., Faculty of Science , Al-Azhar University, Cairo, Egypt.

III- Wastes:

1- **Slaughterhouse wastes (SHW):** The slaughterhouse wastes (SHW) applied in the course of solid-state fermentation (SSF) were collected from different slaughterhouses (SH) of El-Khanka, Cairo, Egypt. The SH- wastes were composed of small intestine , large intestine, some particles from stomach, blood and some visceral wastes of caws and buffaloes , the samples were mixed together, dried in open air and then ground until these particles become smaller and homogenous and then added to the production media.

2- **Fish wastes:** The fish wastes (FW) applied in the course of solid- state fermentation (SSF) were collected from different fish wastes of El-Matarya fish market, Cairo, Egypt. The fish wastes composed of offal wastes (Head, fins, tails, scales, thorns, blood and some particles from skin). The samples were mixed together, dried in open air, ground until these particles become smaller and homogenous and the added to the production media.

3- **Poultry wastes:** The poultry wastes (PW) applied in the course of solid-state fermentation (SSF) were collected also from different poultry wastes of El-Matarya poultry markets, Cairo, Egypt. The poultry wastes composed of internal wastes, heads and feathers. The samples were mixed together, dried in open air the then ground until these particles become smaller and homogenous and then added to the production media.

IV-Buffers: Phosphate buffers: Phosphate buffer was prepared according to Gomori (33).

V- Reagents:

1- **Acid mercuric chloride** (23): It consists of Mercuric chloride,12g; Conc.HCl ,16.0 ml and dist. Water,80ml. Mix the $HgCl_2$ with water add the acid and shake well solution in completed.

2- **Determination of total soluble protein**: The total protein determination was made according to method of Lowery *et al.,* (56) using albumin as a standard protein.

VI- Protease enzyme assay: Protease clearing zone(PCZ) technique: The protease enzyme activity in the present study was measured by the gelatin cup plate clearing zone(GCZ) technique (7). The assay medium (previously mentioned)into each sterile Petri-dish. Which was allowed to cool and solidify. Three cups were made per each plate by a sterile cork borer (10 mm), 0.1 ml of the previously prepared supernatant of the bacterial isolate was introduced into each cup and then plates were incubated for 12 hours at 55 °C. At the end of incubation period, plates were flooded with 10 ml of acidic mercuric chloride solution prepared as in Cowan (23). The clearing zone diameters were recorded. Both their means as well as standard deviations (SD) were calculated and then taken as an indication of proteolytic activities.

VII- Construction of a standard curve for assaying the protelytic activity: The activity of the protease(s) enzymes(s) was estimated in term of mean diameters of clearing zones (mm) using a special standard curve contracted for such a purpose and covering the range of (100-50000 µg/ml) using the so called gelatin cup plate clearing zone(GCZ) technique.

VIII- Standard curve of protein concentration:

The protein concentration was determined by using the method of Lowry *et al*.,(1951). For this purpose, protein (Bovine serum albumin) was used in different concentrations in the range of (0.02-0.24 mg/ml). Protein content was determined after the addition of Folin reagent and the samples were read at 750 nm (Calorimetrically). The obtained optical densities (OD) were translated by the extrapolation of the useful range of the standard curve as mg/ml.

IX- Parameters controlling protease(s) productivity by *B. brevis*, I-4 and *B. brevis* var. *gelationoamylolyticus*, I-2:

1- Effect of temperature: *B.brevis*, I-4 was allowed to grow on DSHW medium while *B.brevis var. gelatinoamylolyticus*, I-2 was allowed to grow on other production media DFW and DPW and then incubated at different temperatures viz. (10,20,30,40,45,50,55,60,65 and 70°C, then the proteinases productivity was determined for each treatment.

2- Effect different substrate concentrations: For this purposes, different concentrations of each waste SHW; FW and PW (1, 2,3,4,5&6 gm/flask).Each flask containing 20 ml of mineral salts medium.

3- Incubation periods: The two most potent bacterial strains were incubated for different period's viz. (12, 24, 48, 60 and 72 hours at 55°C.

4- pH Values: Different pH values covering the range from (5,8-8) were obtained by using phosphate buffer (0.2M) for the production media.

5- Incubation volume: Different inocula sizes of heavy spores suspensions of the two most potent bacterial strains were used. Each one ml of the bacterial suspension contained in case of *B. brevis*, I-4 (0.70×10^{10} CFU/ml), while in case of *B. brevisvar.gelatinoamylolyticus*,I-2 (0.90×10^{10} CFU/ml). The following volumes (ml) were applied

viz. 0.5,1.0,2.0,3.0.4.0 and 5.0 ml/volume per each flask (250 ml capacity).

6- Flask volume: This was performed by using five different flasks of various capacities viz. 100, 250, 500, 1000 and 2000 ml. Each flask containing 20 ml of the production medium.

7- Shaking and static conditions: This was carried out by incubating flasks containing the production media on the shaker (50 rpm). Another flask was incubated under static state at 55°C for 24 hours as a control.

8- Carbon sources: Different carbon source were introduced into the three production media at an equimolecular level located of 2.0 % sucrose (w/v). Arabinose, fructose, galactose, glucose, mannose, maltose, ribose, sucrose and starch were introduced in this test.

9- Nitrogen sources: Different nitrogen sources were introduced to investigate the effect of nitrogen sources viz. ammonium chloride, ammonium citrate, ammonium sulphate, magnesium nitrate, peptone, potassium nitrate, sodium nitrate and urea. This nitrogen sources were introduced at equimolecular nitrogen content equivalent to that located in sodium nitrate.

10- Different amino acids: Aspartic acid, cysteine, glycine, histidine, isoleucine, methionine, L-Proline, serine, tryptophan and tyrosine were added at equimolecular nitrogen content located in sodium nitrate to investigate the effect of amino acids addition to the production media.

11- Elimination of one or more of the ingredients of the mineral salts: For this purpose, the mineral salts of Dox's medium were used.

12- Different metals: Different metals viz. cadmium chloride, cobalt chloride, lead chloride, nickel sulphate and sodium selenite were introduced into the three production media. These metals were applied at five different concentrations viz. (50,100,150,200 and 250 ppm). Finally all the previously testes the three proteases enzymes productions were determined by GCZ techniques measured.

X- Purification of proteases enzymes: The following steps were performed during the course of purification of protease(s) produced by *B.brevis*,I-4 due to the growth on the production medium (DSHW) and *B. brevis var. gelatinoamylolyticus*,I-2 due to the growth on the two production media (DFW and DPW) under (SSF) conditions.

1- Enzymes production and preparation of the optimum conditions for protease(s) production under solid-state fermentation (SSF) conditions on the three media (DSHW; DFW and DPW): At the end of the incubation period, the bacterial growth was done using a sintered glass G-4 filter and then the obtained filtrate was kept into refrigerator as a crude enzymes filtrate according to Ammar *et al*.(8). This step was carried out for all the three cell- free extracts of the media (DSHW; DFW and DPW).

2- Ammonium sulphate fractionation: Precipitation of protein by ammonium sulphate was applied in this procedure because of the following reasons:

a- The enzyme demonstrated a decrease solubility at high salt concentration.

b- Ammonium sulphate has a little deleterious effect on the enzyme.

c- The high solubility of ammonium sulphate.

The chart of Gomori (33) as mentioned by Dixon and Webb (28) was applied to calculate the solid ammonium sulphate to be added to achieve any given concentration of the cell-free filtrate, under investigation. At first, only 100 ml of the cell-free filtrate of each of the three extracts under investigation were first brought to 20 % saturation by gradual addition of solid ammonium sulphate and the precipitated protein was obtained by centrifugation for 15 minutes at 15000 rpm. The obtained pellet was dissolved into 5 ml of (0.2 M) phosphate buffer (pH 7). Centrifugation was repeated and the pellet was re-suspended in order to determine both the enzyme activity and protein content were determined for each separate fraction.

4- Dialysis: This repeated step of purification was carried out to remove the traces of ammonium sulphate. The procedure was performed by introducing the previous precipitate (selected ammonium sulphate ppt) in solution after dissolving it in the buffer into dialysis bag against distilled water for 12 hours. The obtained enzyme preparation was concentrated against sucrose to the available for the application of column chromatography technique.

5- Application of column chromatography technique: The dialyzed- partially purified –enzyme preparation was applied onto a column packed with Sephadex G-200. This was equilibrated with phosphate buffer (0.2 M)(pH7), then eluted with the same buffer. Preparation of the gel column and the fractionation procedure was carried out as mentioned by Ammar (5).

XI- Characterization and properties of the purified proteases graduation by *B. brevis*, I-4 and *B. brevis var. gelatinoamylolyticus*, I-2:

1- Enzyme concentration: This experiment was performed to investigate the effect of different concentrations of protease enzyme of their activates, The purified proteases enzymes were applied at different concentrations viz. 0.025, 0.050, 0.075, 0.100, 0.125 and 0.150(ml).

2- Substrate (gelatin) concentrations: The substrate (gelatin) was applied at concentrations of (w/v %): 0.075, 0.1, 0.25, 0.5, 0.75, 1.0, 1.5, 1.75 and 2.0.

3- Incubation temperatures: This experiment was carried out by incubating the reaction mixtures (Enzyme and Buffer) at different temperatures viz. 20, 30,40,45,50,55,60,65 and 70°C.

4- pH values: The reaction mixtures (Enzymes and Buffer) were incubated at different pH values viz. 5.7;6.0;6.5;7.0;7.5 and 8.0 using phosphate buffer (0.2M).

5- Incubation periods: The reaction mixtures were incubated for 6,12,18,24,36,48,60 and 72 hours. Proteases activates were performed as described before.

6- Activators and /or inhibitors: Cadmium chloride; calcium chloride; lead chloride; nickel sulphate and magnesium sulphate were added at different concentrations viz. 100,200,300,400 and 500 ppm. Proteases activity was performed for each treatment as described before.

XII- Application of the three crude proteases enzymes and purified proteases enzymes in the industrial processes:-

1- The use of the three crude purified proteases enzymes in leather industry: Several different proteases can be used in the invading stages of the manufacturing of leather process. The use of proteases enzymes lies in the fact that protein is the major building block of hair and skin.

The proteases enzymes used in the beating of leather, where the wool of the hides and skin of animals was removed and then treated with protease enzymes and chemicals to make them soft and supple. In this experiment, the cow's leather was treated with Grease (as in the traditional processes) and compared with another cow's leather treated with the crude proteases enzyme under investigation. At the end of experiment, the leather pieces were compared with each other to investigate their softness.

2- The use of the three purified proteases enzymes in detergent industry:

This experiment was made by comparing the purified protease with an industrial detergent (OMO detergent). The comparison was carried out by testing the ability of the purified enzymes protease to remove the chocolate stain. The same experiment was carried out by using the OMO detergent. Three different temperatures were used for comparison, 20 (cold water), 40 and 55°C for both the purified protease enzymes and OMO detergent. At the end of experiment, the chocolate stains were experimented and compared with each other.

Results

1- Selection of the screening organisms that allowed to grow on the production media for production of protease:

Five different thermophiles bacterial strains were allowed to grow on the three different media to evaluate their proteolytic activities. The best organism(s) that showed the highest productivity for each media was selected for further investigations. The proteolytic activates were carried out , using the gelatin- cup plate clearing zone(GCZ) technique (Ammar *et al*., 1991). The results were recorded in table (1). It was obvious that, *B. brevis*, I-4 was found to be the best organism for maximum production of protease enzyme when allowed to grow on DSHW medium, while *B.brevis var.gelatinoamylolyticus*,I-2 was the best organism for maximum production of protease enzyme when allowed to grow on DFW and DPW media.

Table (1): A quantitative screening test of the protease productivity using five different bacterial isolates grown on the three different production media and incubated at 55°C under SSF conditions.

Thermophilic bacterial isolates	Protease productivity (unit/ml).		
	DSHW	DFW	DPW
B. brevis I-4	662.62±0.00	437.55±0.00	347.56±2.0
B. brevis var. gelatinoamylolyticus I-2	437.55± 0.00	502.37±0.33	437.55±0.00
B. brevis var.gelatinoxyloureamylolyticus I-5	399.05±0.28	276.07±0.33	251.79±0.20
B.brevisureamylolyticus I-7	417.85± 0.20	302.71±0.20	276.07±020
B.brevisxyloamylolyticus I-8	437.55±0.00	289.08±0.00	170.22±0-00

2- Parameters regulating the biosynthesis of proteases by *B.brevis*,I-2 and *B. brevis* var. *gelatinoamylolyticus*,I-2 grown under solid state fermentation (SSF) conditions:

Factors were investigated for their effects on the process of proteases production viz. Effect of different incubation temperatures (10-70°C), effect different substrate concentrations (1-6 g/flask), effect of different incubation periods (12-72 hours), pH values (5.8-8), incubation volume (0.5-5.0 ml), different flask volume (100-2000ml), different incubation conditions (Shaking and static conditions), different carbon sources (Arabinose, Fructose, Galactose, Glucose, Mannose, Maltose, Ribose, Sucrose and Starch), different nitrogen sources (Ammonium chloride, Ammonium citrate, Ammonium sulphate, Magnesium nitrate, Peptone , Potassium nitrate, Sodium nitrate and Urea), different amino acids (Aspartic acid , Cystine, Glycine, Histidine, Isolucine, Methionine, L-Proline, Serine, Tryptophan and Tyrosine), elimination of one or more of the ingredients of the mineral salts used in the production media and finally different metals (activators and inhibitors) viz. Cadmium chloride, Cobalt chloride, Lead chloride, Nickel sulphate and Sodium selenite) published previously (Ammar *et al*., 2003). A summary of the optimal conditions of protease production using three production media by *B.brevis*, I-4 and *B. brevis var. gelatinoamylolyticus*, I-2 under SSF conditions presented in table(2).

Table (2): A summary of the optimal conditions of protease productivity by *B. brevis*, I-4 and *B. brevis var. gelatinoamylolyticus*, I-2 using the three production media under (SSF) conditions.

No.	Optimal parameters	Optimum proteases production conditions		
		DSHW	FW	PW
1	Incubation temperatures	55	55	55
2	Substrate concentration (g)	2	2	2
3	Incubation period (Hours)	24	24	24
4	pH Values	7	7	7
5	Incubation volume (ml)	1	1	1
6	Flask volume (ml)	250	250	250
7	Incubation conditions	Static	Static	Static
8	Carbon source (2 g/100 ml)	Starch	Starch	Starch
9	Nitrogen source	Sodium nitrate	Potassium nitrate	Potassium nitrate
10	Amino acid	Glycine	L-Proline	L-Proline
11	Mineral salts	$NaNO_3$& KH_2PO_4	KH_2PO_4 &$MgSO_4.7H_2O$	KH_2PO_4 &$MgSO_4.7H_2O$
12	Metal ions (200ppm)	Sodium selenite	Sodium selenite	Sodium selenite

3- Purification of thermostable proteases enzymes produced by *B. brevis*, I-4 and *B. brevis var. gelatinoamylolyticus*, I-2 under SSF condition at 55°C.

The proteases enzymes produced under the optimum conditions previously determined was purified by applying ammonium sulfate fractionation, dialysis flowed by column chromatography on Sephadex G-200 and Sephadex G-100. Purification steps were as the following:

Step (1): Enzyme production and preparation of cell-free filtrate (CFF). *B. brevis*, I-4 allowed to grow on fish wastes (FW) and Poultry wastes (PW) under all the optimal conditions previously mentioned in table (2). At the end of the incubation period, the obtained extracts were centrifuged at 15,000 r.p.m. for 15 minutes at 10°C and the resulted supernatants i.e. (700ml), for the three media were tested for their proteolytic activity and protein content. This resulted in having specific activity (6262.71 units/ mg. protein.ml) for SHW protease, whereas (5233.71 Units/mg. protein.ml) for FW protease, and (4773.19 Units/mg. protein.ml) for PW protease. The obtained cell-free filtrate (CFF) were kept as stock enzymes in the refrigerator for further steps of purification.

Step(2): Fractional precipitation with ammonium sulphate: Only 100ml of the stock cell free filtrates were applied to five different concentrations of ammonium sulphate: 20;40;60;80 and 100%. The results were listed in table (3). Results indicated that the most active enzyme preparation could be obtained at 60% ammonium sulphate concentration. After centrifugation, the obtained precipitation were dissolved in 5 ml phosphate buffer (0.2M) at pH7. Both enzyme activity and protein content were determined for the three different enzymes, which are corresponding to the specific activity. Then, the enzymes were subjected to a process of dialysis against distilled water to get rid of the excess ammonium sulphate. The remained stock of the cell-free extract i.e. 600 ml was applied to ammonium sulphate precipitation at the centrifugation level of 60%. Only 45 ml were obtained at the end of the process of dialysis against the distilled water for 12 hours.

Step(3): Concentration by dialysis against sucrose: The most active ammonium sulphate fractions previously obtained at 60% concentration (45ml) were dialyzed against sucrose until a volume of (4 ml) was obtained in case of the three purified enzymes. The three enzymes recorded specific activities (55819.66; 37987.18 and 28816.18 Units/mg. Protein.ml) in case of SHW protease, FW protease and PW protease respectively. At this stage, the purification fold of enzymes were (8.9; 7.25 and 6.0) in case of SHW protease, FW protease and PW protease respectively as shown in table (6, 7and 8).

Step (4): Purification of SHW protease, FW Protease and PW protease enzymes on Sephadex G-200 column chromatography: After allowing the Sephadex gel column to be settled, one ml of the enzyme SHW obtained from the previous step was added to the upper surface of the gel column. Phosphate buffer(0.2 M) at pH 7.0 was used for eluting and the 50 fractions were collected in the form of 5 ml aliquots. Enzyme activity and protein content were determined in each fraction and the corresponding specific activity was calculated. The same steps were repeated for FW protease and PW protease. Results of these steps were recorded in table (4) and represented graphically in fig.(1)in which only one peak was obtained in case of the three enzymes. These peaks were at fractions number (18) in case of SHW protease, and (20) in case of both FW protease and PW protease. At this stage, the purification folds of protease were up to 11.0 in case of SHW protease, while 8.5 in case of FW protease and 7.41 in case of PW protease enzymes.

Step (5): Purification of SHW protease, FW protease and PW protease enzymes on Sephadex G-100 column chromatography:

The active fractions of the peak obtained after applying the first column of Sephadex G-200 were mixed together, concentrated and then applied onto a second Sephadex G-100 column. Fifty fractions each of 5 ml aliquots were eluted and both proteolytic activity and protein content for each fraction were determined in case of the three enzymes. Thus, the results that were recorded in table (5) and represented graphically in fig. (2) the second purification stage of SHW protease ; FW protease and PW protease enzymes on Sephadex G-100 column chromatography. As shown in table (5) only one peak was obtained in case of the three enzymes. The most active fraction lied between fractions number (6-12) in case of both SHW protease and FW protease, while between fractions number (6-11) in case of PW protease enzymes. At these stages, the purification folds of enzymes were (16.83; 14.45 and 14.16) in case of SHW protease, FW protease and PW protease enzymes respectively.

A summary of purification steps of SHW protease, FW protease and PW protease enzymes produced by *B. brevis*, I-4 and *B. brevis var. gelatinoamylolyticus*, I-2 under SSF conditions were recorded in table (6, 7 and 8).

Table (3): Ammonium sulphate fractionation pattern of protease enzyme produced by *B. brevis*, I-4 and *B. brevis var. gelatinoamylolyticus*, I-2 under SSF conditions.

Amm. Sulphate Conc,(%)	Enzyme activity (Unit/ml)			Protein content (mg)			Specific activity (Units/mg protein/ml)		
	SHW	FW	PW	SHW	FW	PW	SHW	FW	PW
Crude enzyme	1321.38±0.00	1099.08±0.00	1002.37±0.20	0.211	0.21	0.21	6262.46	5233.71	4773.19
0-20	1741.92±0.00	1588.65±0.28	1448.87±0.20	0.2	0.21	0.2	8709.6	7562	7244.35
20-40	2000.0±0.00	1909.98±0.00	1741.92±0.00	0.23	0.19	0.19	8695.65	10052.52	9168
40-60	3169.78±0.00	2670.76±0.20	2517.85±0.00	0.16	0.15	0.15	19811.12	17805.06	16785.66
60-80	1049.61±0.00	1002.37±0.20	347.55±0.00	0.15	0.14	0.13	6997.4	7159/78	2673.46
80-100	726.15±0.28	632.45±0.00	347.56±0.28	0.14	0.12	0.11	5186.78	5270.41	3159/63

Table (4): Fractionation pattern of (SHW) protease (FW) protease and (PW) protease enzymes using produced by *B. brevis*, I-4 and *B. brevis var. gelatinoamylolyticus*, I-2 Sephadex G-200 column chromatography.

Fractionation no.	Protease activity (unit/ml)			Protein content (mg/ml)			Specific activity (unit/mg protein/ml)		
	SHW	FW	PW	SHW	FW	PW	SHW	FW	PW
1-13	0.0	0.0	0.0	ND	ND	ND	0.0	0.0	0.0
14	30.97± 0.00	0.00±0.00	0.00±0.00	0.005	0.00	0.00	6194	0.00	0.00
15	47.97±0.00	0.00±0.00	0.00±0.00	0.006	0.00	0.00	7995	0.00	0.00
16	74.30±0.00	20.00±0.28	20.00±0.2	0.008	0.005	0.005	9287.5	4000	4000
17	93.54±0.00	38.90±0.00	24.60±0.00	0.006	0.007	0.006	15590	5557.14	4100
18	276.07±0.00	60.39±0.00	32.43±0.00	0.004	0.008	0.007	69017.5	7548.75	4632.85
19	200.0±0.28	115.08±0.00	74.30±0.20	0.011	0.008	0.008	18181.8	14385	9287.5
20	74.30±0.00	178.25±0.00	141.5±0.00	0.016	0.004	0.004	4643.75	44562.5	35375
21	47.97±0.00	74.30±0.00	74.30±0.20	0.013	0.011	0.006	3690	6754.54	7995
22	12.6± 0.28	30.97±0.00	21.82±0.00	0.012	0.012	0.005	1050	2580.83	4364
23	0.0	24.60±0.00	21.61±0.00	0.0	0.013	0.011	0.0	1892.30	1964.45
24	0.0	20.0±0.00	8.43±0.00	0.0	0.011	0.011	0.0	1818.18	766.36
25-50	0.0	0.00±0.00	0.0	ND	ND	ND	0.0	0.0	0.0

Table (5): Fractionation pattern of (SHW) protease (FW) protease and (PW) protease enzymes using produced by *B. brevis*, I-4 and *B. brevis var. gelatinoamylolyticus*, I-2 Sephadex G-100 column chromatography.

Fractionation no.	Protease activity (unit/ml)			Protein content (mg/ml)			Specific activity (unit/mg protein/ml)		
	SHW	FW	PW	SHW	FW	PW	SHW	FW	PW
1-5	0.00	0.00	0.00	ND	ND	ND	0.00	0.00	0.00
6	115.08±0.00	79.80±0.00	30.97±0.20	0.01	0.007	0.007	11508	11400	4424.28
7	141.58±0.00	115.08±0.20	100.25±0.00	0.008	0.007	0.005	17697.5	16440	20050
8	170.20±0.00	141.58±0.00	263.65±0.28	0.006	0.006	0.0039	28366.6	23596.66	67602.56
9	347.94±0.00	302.71±0.00	115.08±0.00	0.0033	0.004	0.005	105436.36	75677.5	23016
10	178.25±0.00	115.08±0.00	60.39±0.00	0.006	0.005	0.007	29708.33	23016	8625.14
11	93.54±0.00	74.30±0.33	30.97±0.00	0.01	0.01	0.011	8503.63	7430	2815.45
12	12.05±0.00	8.43±0.00	0.00	0.011	0.00	0.00	1004.16	766.36	0.0
13-50	0.00	0.00	0.00	ND	ND	ND	0.00	0.0	0.0

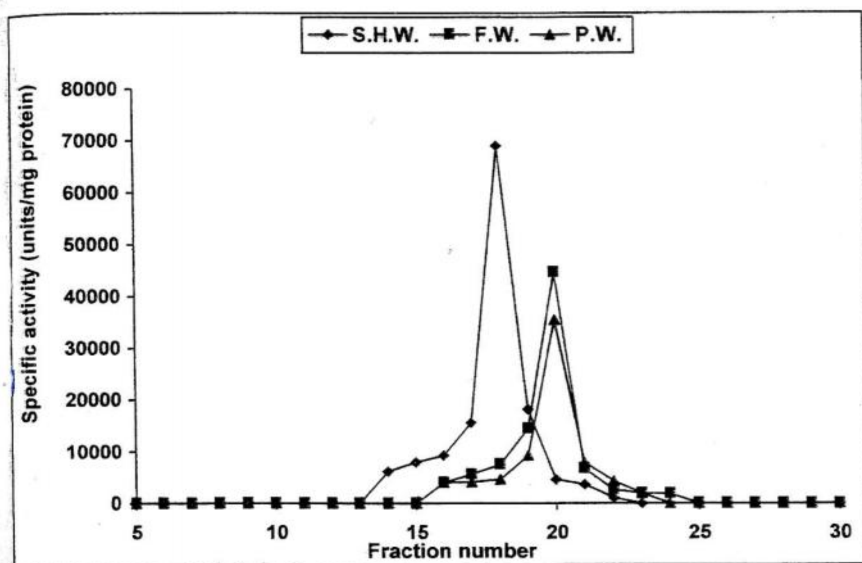

Fig.(1): Fractionation pattern of SHW protease (FW) protease and PW protease enzymes produced by *B. brevis*,1-4 and *B.brevisvar.gelatinoamylolyticus*, 1-2 using Sephadex G-200 column chromatography.

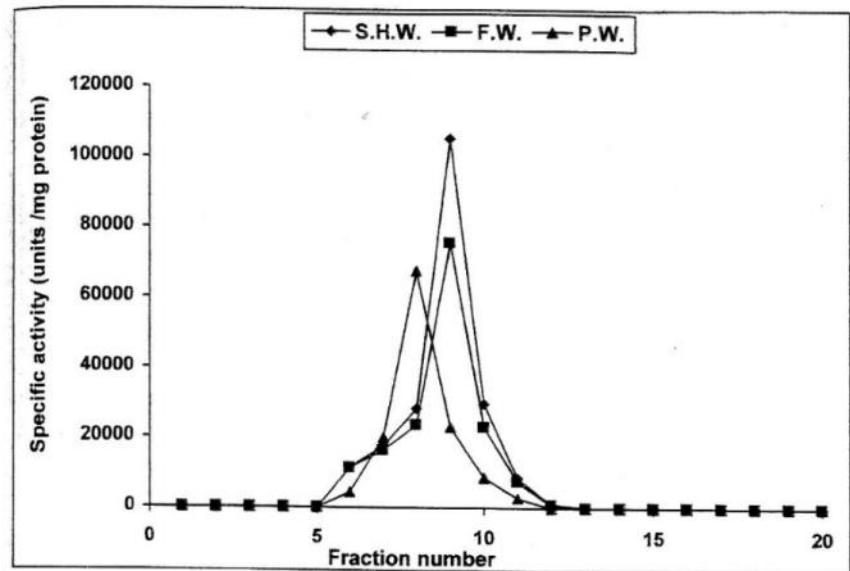

Fig.(2): Fractionation pattern of SHW protease (FW) protease and PW protease enzymes produced by *B. brevis*,1-4 and *B.brevisvar.gelatinoamylolyticus*, 1-2 using Sephadex G-100 column chromatography.

Table (6): A summary of purification steps of protease enzyme produced by *B. brevis*, I-4 allowed to grow on (DSHW) under SSF conditions.

Step no.	Purification steps	Total volume (ml)	Enzyme activity (unit/ml)	Protein content (mg/ml)	Total enzyme activity (X)	Total protein activity (y)	Specific activity (x/y) units /mg. Protein/ml)	Purifications folds
1	Cell free filtrate (CCF)	700	1321.38	0.211	924966	147.7	6262.46	1
2	Ammonium sulphate fractionation (60%)	100	3169.78	0.16	316978	16	19811.12	316
3	Dialysis against sucrose	4	5023.77	0.09	20095.08	0.36	55819.66	8.9
4	Sephadex G-200 First step purification	5	276.07	0.004	1380.35	0.02	69017.5	11
5	Sephadex G-100 Second step purification	5	347.94	0.0033	1739.7	0.016	105436.36	16.83

Table (7): A summary of purification steps of FW protease enzyme produced by and *B. brevis var. gelatinoamylolyticus*, I-2 allowed to grow on (DFW) under SSF conditions.

Step no.	Purification steps	Total volume (ml)	Enzyme activity (unit/ml)	Protein content (mg/ml)	Total enzyme activity (X)	Total protein activity (y)	Specific activity (x/y) units /mg. Protein/ml)	Purifications folds
1	Cell free filtrate (CCF)	700	1099.08	0.21	769356	147	5233.71	1
2	Ammonium sulphate fractionation (60%)	100	2670.76	0.15	267076	15	17805.06	3.4
3	Dialysis against sucrose	4	4178.59	0.11	16714.36	0.44	37987.18	7.25
4	Sephadex G-200 First step purification	5	178.25	0.004	891.25	0.02	44562.5	8.51
5	Sephadex G-100 Second step purification	5	302.71	0.004	1380.35	0.02	75677.5	14.45

Table (8): A summary of purification steps of (PW) protease enzyme produced by and *B. brevis var. gelatinoamylolyticus*, I-2 allowed to grow on (DPW) under SSF conditions.

Step no.	Purification steps	Total volume (ml)	Enzyme activity (unit/ml)	Protein content (mg/ml)	Total enzyme activity (X)	Total protein activity (y)	Specific activity (x/y) units /mg. Protein/ml)	Purifications folds
1	Cell free filtrate (CCF)	700	1002.37	0.21	701659	147	4773.19	1
2	Ammonium sulphate fractionation (60%)	100	2517.85	0.15	251785	15	16785.66	3.51
3	Dialysis against sucrose	4	3169.78	0.11	12679.12	0.44	28816.18	6.0
4	Sephadex G-200 First step purification	5	141.5	0.004	707.5	0.02	35375	7.41
5	Sephadex G-100 Second step purification	5	263.65	0.0039	1318.25	0.0195	67602.56	14.16

4-Properties of the purified SHW, FW and PW proteases enzymes produced under SSF conditions:

These properties include the effect of enzyme concentration, substrate concentration, temperature, pH and incubation period.

1- **Enzyme concentration:** Results recoded in table (9) exhibited a continuous increase of the enzymes activity due to the corresponding increase of their concentration. This increase in protease activity reached its maximum at (0.125ml) protease enzyme concentration.

2- **Substrate(Gelatin) concentration:** In this experiment, different concentrations of gelatin were used. Results were recorded in table (10). The increase of substrate (gelatin) concentration over 0.1% decreased the activity of SHW, FW and PW proteases enzymes, which was estimated by GCZ technique. Thus, the optimal substrate concentration was 0.1% under the given conditions (Table 10). Such suppressive effect of high substrate concentration on enzyme activity was known as early as 1913 when Michaelis and Menten(59) stated that within sufficiently high substrate concentration, the rate of enzyme action was actually slowed. Furthermore, West *et al*., (104) stated that the rate of enzyme action did not increase beyond a certain value of substrate concentration.

3- **Incubation temperature:** the results listed in table (11) showed that, the optimal temperature for the three proteases enzymes activities was 55 ºC. The temperature below or above these particular temperature had deleterious effects on the enzymes activity.

4- **pH values:** Preparation of phosphate buffer (0.2M) was as previously mentioned in the section of materials and methods. Results were recorded in table (12). It was obvious that the optimum pH was pH 7.0 for SHW, FW and PW proteases enzymes activates.

5- **Incubation periods:** The three purified enzymes were incubated for different periods ranging between (12-72 hours) at 55 ºC. Results recorded tin table (13), showed that the enzymes activates increased with the increase of the incubation period reaching its maximum at 60 hours and then the enzymes activates become constant.

Table (9): Different concentrations of the purified SHW, FW and PW protease enzymes in relation to their activities.

Protease concentration (ml)	Protease activity (unit/ml)		
	SHW	FW	PW
0.025	115.08±0.00	81.41±0.00	60.39±0.00
0.050	178.25±0.20	141.58±0.00	74.30±0.00
0.075	437.55±0.00	347.56±0.33	120.51±0.28
0.100	662.62±0.20	603.99±0.33	399.05±0..20
0.125	1049.61±0.28	833.73±0.00	662.62±0.00
0.150	1049.61±0.28	833.73±0.00	662.62±0.00

Table (10): Different substrate (gelation) concentrations in relation to the activity of the purified SHW, FW and PW protease enzymes.

Substrate (gelatin) concentration (g/ml)	Protease activity (unit/ml)		
	SHW	FW	PW
0.075	833.73± 0.20	632.45±0.0	526.05±0.0
0.1	1002.37±0.20	833.73±0.0	662.62±0.00
0.25	833.73±0.00	662.62±0.0	399.05±0.00
0.50	662.62±0.00	437.55±0.0	178.25±0.33
0.75	302.71±0.20	276.07±0.0	141.58±0.00
1.0	219.28±0.00	190.89±0.28	115.08±0.00
1.25	200.0±0.33	170.22±0.28	115.08±0.20
1.50	141.58±0.00	100.23±0.0	93.58±0.28
1.75	115.08±0.00	70.96±0-20	50.23±0.33
2	74.30±0.00	60.39±0.00	38.99±0.00

Table (11): Different incubation temperatures in relation to the activity of the purified SHW, FW and PW protease enzymes.

Temperature (ºC)	Protease activity (unit/ml)		
	SHW	FW	PW
10	0.00±0.00	0.00±0.00	0.00±0.00
20	8.14±0.20	0.00±0.00	0.00±0.00
30	20.00±0.00	8.12±0.00	0.00±0.00
40	74.30±0.28	47.97±0.0	60.39±0.33
45	276.07±0.00	126.09±020	81.41±0.20
50	726.15±0.20	437.55±0.0	437.55±0.0
55	1049.61±0.00	833.73±0.0	662.62±0.00
60	115.08±0.33	79.80±0.33	81.41±0.33
65	47.97±0.00	38.99±0.00	38.99±0.00
70	0.00±0.00	0.00±0.00	0.00±0.00

Table (12): Different pH values in relation to the activity of the purified SHW, FW and PW protease enzymes.

pH values (Phosphate buffer) (0.2M)	Protease activity (unit/ml)		
	SHW	FW	PW
5.7	56.76±0.23	38.99±0.00	38.99±0.00
6.0	175.08±0.00	104.96±0.28	74.30±0.28
6.5	347.55±0.0	276.07±0.20	251.79±0.20
7.0	1049.61±0.0	833.73±0.00	662.62±0.00
7.5	170.22±0.33	141.5±0.00	100.23±0.28
8	93.54±0.00	74.30±0.00	50.23±0.33

Table (13): Different incubation period in relation to activity of the purified SHW, FW and PW proteases.

Incubation period (hours)	Protease activity (unit/ml)		
	SHW	FW	PW
6	10.25±0.00	8.14±0.00	8.14±0.00
12	437.55±0.20	363.94±0.28	115.08±0.28
18	833.73± 0.00	632.45± 0.20	437.55±0.00
24	1049.61±0.00	833.73± 0.00	662.6±0.00
36	2000.0± 0.28	1588.65±0.00	1383.38±0.33
48	5768.06± 0.00	3639.40±0.33	3169.78±0.00
60	7261.56±0.00	5768.06± 0.00	5260.53± 0.20
72	7261.56±0.20	5768.06±0.00	5260.53±0.20

5-Application of the thermostable proteases enzymes:

1- Application of the three crude proteases enzymes in the leather bating process in comparison with the white grease commercial method:

Eight pieces (5cm ×5cm) of cow's neck leather were selected. One of them was left untreated as control 1 and another piece of leather was treated with the traditional white grease (1 gm dissolved in 100 ml) as control 2. While each two of the other six dissolved were treated with one of the three crude enzymes (0.2 ml) dissolved in 100 ml to water under investigation viz. SHW; FW and PW proteases enzymes.

It was obvious that the leather pieces treated with the different proteases enzymes were found to be more soft than the leather piece treated with the commercial white grease in comparison with control (untreated leather piece).

2- Applying of the three purified thermostable neutral proteases in the detergents industry:

Small square pieces (5cm ×5cm) of new cotton fabric were selected. One of them was left without stain as control 1, while the other pieces were stained with chocolate stains. After drying, one piece was left without treatment as control 2. On the other hand, one piece of cloth with chocolate stain was treated with OMO detergent (1gm dissolved in 100 ml tap water) in comparison with other three cloth pieces that were treated the three different purified proteases (02ml dissolved in 100 ml tap water separately. Then these stained cloth pieces were washed at different temperature values. The results showed different degrees of clearing as follows:

i- Washing in cold water at 20ºC for 30 minutes: In comparison with control (1&2) , the stained cloth pieces treated with the three purified proteases enzymes at 20 ºC for 30 minutes, were found to be more clean than the cloth piece treated with OMO detergent under the same conditions (plate1).

ii- Washing in water at 40ºC for 30 minutes: In comparison with control (1&2) , the cloth treated with OMO detergent , showed a degree of cleaning less than the cloth pieces that were treated with the three purified protease enzymes under investigation specially SHW protease (Plate2).

iii- Washing in water at 55ºC for 30 minutes: The results showed that the highest degree of cleaning at 55ºC was achieved by applying the purified SHW protease enzyme in comparison with OMO detergent, and if they in turn were compared with control (1&2)(Plate3).

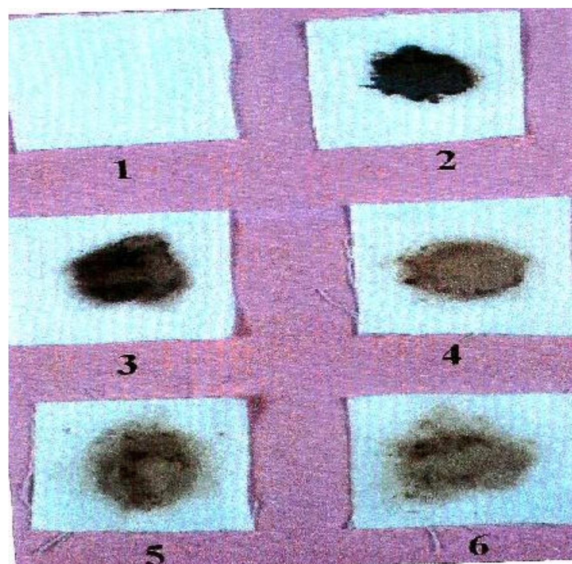

Plate (1): (1) Unstained cloth piece as control1; (2) Cloth piece with chocolate stain as control 2; (3) Cloth piece with chocolate stain when treated with OMO detergent, (4;5&6) Cloth pieces with chocolate stain when treated with the three purified enzymes SHW;FW and PW proteases respectively. All treatment were performed at 20ºC for 30 minutes.

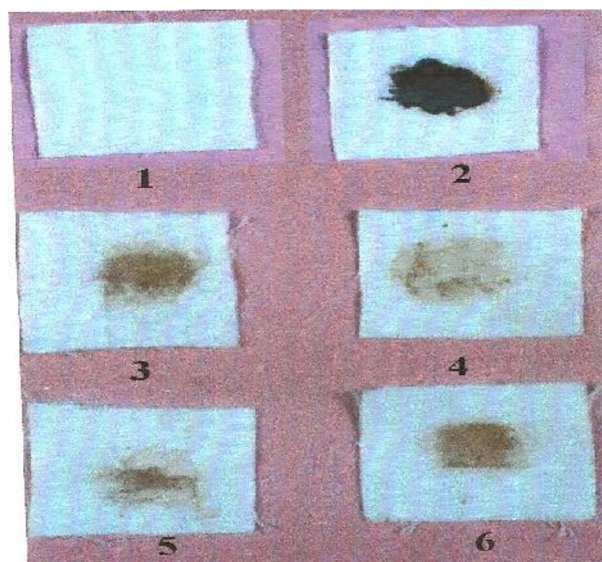

Plate (2): (1) Unstained cloth piece as control1; (2) Cloth piece with chocolate stain as control 2; (3) Cloth piece with chocolate stain when treated with OMO detergent, (4;5&6) Cloth pieces with chocolate stain when treated with the three purified enzymes SHW;FW and PW proteases respectively. All treatment were performed at 40ºC for 30 minutes.

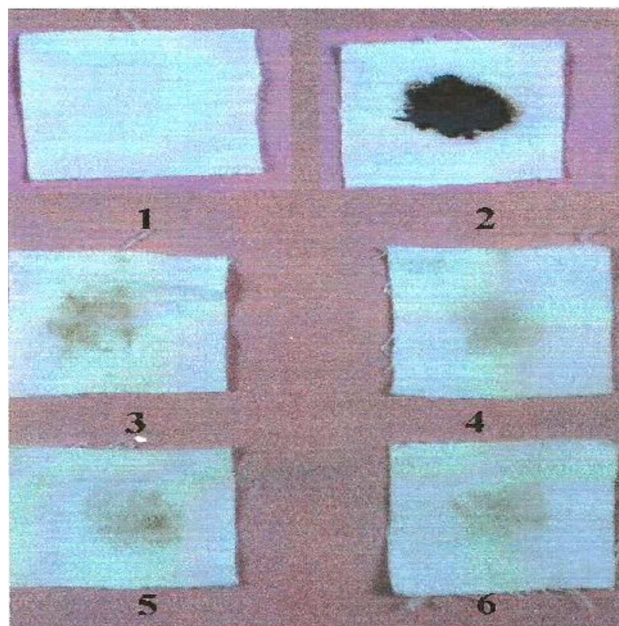

Plate (3): (1) Unstained cloth piece as control1; (2) Cloth piece with chocolate stain as control 2; (3) Cloth piece with chocolate stain when treated with OMO detergent, (4;5&6) Cloth pieces with chocolate stain when treated with the three purified enzymes SHW;FW and PW proteases respectively. All treatment were performed at 55ºC for 30 minutes.

Discussion

Enzymes are biological catalysts that facilitate the conversion of substrates into products by providing favorable conditions that lower the activation energy of the reaction. An enzyme may be a protein or a glycoprotein and consists of atleast one polypeptide moiety. Proteases are the most important industrial enzymes and comprise about 25% of commercial enzymes in the world (4; 70).

Proteases represent an essential group of enzymes that are widely produced and used industrially, thus the need for new microbial isolates with new features is of utmost necessity for industrial applications. Proteases represent an important group of enzymes that are used in various fields, covering a wide range of industrial applications, such as food, detergent, tannery, chemical and pharmaceutical industries (54).

In addition, for an enzyme to be used in detergents, it should be stable at high temperature and active in the presence of other detergent ingredients, such as surfactants, bleach activators, bleaching agents, fabric softeners and other formulation substances (10).

Microorganisms constitute the major source of proteases, including both extracellular and intracellular ones (20).Fermentation is a method of generating enzymes for industrial purposes. It involves the use of microorganisms like bacteria, fungi and yeast to produce the enzymes. Two methods of fermentation have been used to produce enzymes. These are submerged fermentation and solid-state fermentation. Submerged fermentation involves the production of enzymes by microorganisms in a liquid nutrient media. Solid-state fermentation is the cultivation of microorganisms and enzyme production on a solid substrate. Renge *et al.,* (79) reported that the SSF uses inexpensive substrates and yields higher volumetric productivity. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry (97).

Solid–state fermentation (SSF) processes is probably the largest enzyme procedure on a worldwide basis. The microorganisms in solid-state cultivation grow under conditions closer to their natural habitat, they may be more capable producing certain natural habitat, and they may be more capable of producing certain enzymes and metabolites , which are usually not to be produced in submerged cultures discussed the advantages and disadvantages of solid substrate fermentation compared with liquid medium fermentation. Moreover, solid–state fermentation is an economical and simple method for the production of industrial enzymes.

During the last decades, microbial proteases attracted the attention of many workers. The aim of this work was to investigate the productivity of thermostable proteases enzyme produced by microorganisms (thermophilic bacteria) while attacking different natural substrate under SSF conditions. Substrates play an important role in production of enzymes using microbial source. A novelty in fermentation medium design is necessary to increase protease enzyme production with low cost solid substrates. The enzymes under investigation were produced by *B. brevis*,I-4 and *B. brevis var. gelatinoamylolyticus*, I-2 that were isolated from fresh water hyacinth collected from Auseem area, one of the most famous districts belonging to El-Giza Governorate, Egypt, isolated and identified by Omar (69). These bacteria were found to be important not only for protease (s) production but also for attacking three different substrate , viz. slaughter house wastes (SHW) , fish wastes (FW) and poultry wastes (PW) by acting on the protenaceous components of these wastes.

Members of the genus *Bacillus* are widely used in industry in the large-scale production of enzymes, particularly proteases (62).Among the various bacterial spp., interest in *Bacillus* spp. has gained much importance, as their enzyme complement is robust enough to suit the relatively hostile industrial process conditions. *Bacillus* spp. accounts for 35% of total microbial enzyme sale (48;88). Thus, *Bacillus* spp. are considered super secreting bio-factories for production of various industrial products including enzymes (27;75; 43).

Karn and Karn (50) evaluate and characterize of protease production by *Bacillus* sp. induced by UV – mutagenesis. El-Eskafy *et al*., (29) produced thermostable protease from *Bacillus amyloliquefaciens* as Egyptian marine isolate. The ability of such proteolytic bacteria to develop and utilize proteinaceous substrates, their potentiality to produce proteases, factors affecting their productivity, purification and properties of their proteases are the main target for their possible use on both biodetergent and leather industries.

In the present investigation, the three different substrates (SHW, FW and PW) were attacked by thermophilic bacteria *Bacillusbrevis*,I-4 and *Bacillusbrevisvar. gelatinoamylolyticus*,I-2 to produce the thermostable proteases. Fortunately, data indicated that the production media used gave relatively high yield of proteases enzymes.

Usharani (94) isolated protease from *B. latrosporus* had a maximum of extra cellular protease enzyme activity was at 3 days. Zambare (105) also found *Bacillus* sp. for protease production and maximum activity of 125.68U/ml at the end of 48 hr of incubation at 40˚C.

Enzyme production by microorganisms is greatly influenced by media components, especially carbon and nitrogen sources, and physical factors such as temperature, pH, and incubation time and inoculums density (64). It is important to produce the enzyme in inexpensive and optimized media on a large scale for the process to be commercially viable(64).

Thermo-stable proteases are advantageous in some applications because higher processing temperature can be employed, resulting in faster reaction rates, increase in the solubility of nongaseous reactants and products and reduced incidence of microbial contamination by mesophilic organisms. Proteases secreted from thermophilic bacteria are thus of particular interest and have become more useful in a range of commercial applications (78; 2; 98; 41).

However, the present results are in accordance with those reported by Pravin *et al*., (74);Tavea *et al*.,(92); Thirumala and Vishnuvardhan(93);Badhe*et al*.,(12) and Qureshi *et al*., (88).

It was worthy to investigate some factors that affect the productivity of proteases by *Bacillusbrevis*,I-4 and *Bacillus brevisvar. gelatinoamylolyticus*,I-2. These factors indicated the parameters affected the proteases production (6). Maximum enzyme production time varies among different *Bacillus* spp(88; 42; 14) and depends on microbial strain, cultural and environmental conditions and the genetic potential of the organism. Growth and metabolite (enzyme) production may be partially or completely associated (65; 24).

Bacillus spp. have the capability to efficiently utilize various substrates for the economic production of commercial products thereby making them workhorses for industrial processes. There are reports where *Bacillus* spp. are known to utilize crude substrates for protease production. Corn-starch as a carbon source was found to increase enzyme production from *Bacillus* sp. RKY3. The type and availability of nitrogenous precursors in the medium influence the production of extracellular enzymes. *B. subtilis* K-1 produced maximum protease when soybean meal was used as nitrogen source while *Streptomyces* sp. DP 2 did so with mustard cake. Besides, nitrogen source can significantly affect the medium pH during the course of fermentation, which in turn may influence enzyme activity and stability (51; 92).

Thermostability refers to prolonged stability of enzyme at high temperatures. The thermostability mechanisms for thermozymes are varied and depend upon molecular interactions such as hydrogen bonds, electrostatic and hydrophobic interactions, disulfide bonds, and metal binding which can promote a superior conformational structure for the enzyme (15; 93).

Incubation period, pH, incubation temperature and size of inoculum are important parameters for maximum yield of enzyme varies among various species or even in the same species isolated from various sources. Many workers have reported a broad incubation period ranging from 36 to 96 h for the maximum yield of protease enzyme by *Bacillus* strains (36; 37; 67).

Initial pH of the medium required for obtaining maximum production depends not only upon the bacterium but also upon the ingredients of the medium. Maximum alkaline protease production has been reported from different pH ranges (7-11) by different *Bacillus* species (52). Temperature is one of the most critical parameters that have to be controlled in any bioprocess (22). Optimum production temperature for different *Bacillus* species have been reported as, 25° C for *Bacillus circulans* (46;47;34).

A trial was given in order to obtain the purified proteses enzymes produced by *Bacillusbrevis*,I-4 and *Bacillusbrevis var. gelatinoamylolyticus*,I-2 from the filtrates of the three different production media under investigation and to create an interesting comparative study of the characteristics of the three purified enzyme preparations was accomplished. Therefore, the purification of the crude enzymes were carried out. The purification procedure included cell free filtrate preparation. Ammonium sulphate fractionation, dialysis and applying Sephadex G-200 column followed by Sephadex G-100 column chromatography technique.

Similarly, Asker *et al*. (10) demonstrated that *Bacillus megatrium* protease enzyme possess a specific activity of 41.09 U/mg after purification usingammonium sulfate. However, the yield of the enzyme after purification was found to be low. This might bedue to the autolysis of the enzyme in each purification step.

Mushtaq *et al*., (63) used three microbial cultures *Bacillus subtilis* DSM 1970, *Bacillus subtilis* GCU-8 and *Bacillus licheniformis* DSM 1969were screened for protease production by casein agar plate method. Among these *Bacillus subtilis*GCU-8 was found to be the most potent protease producer in wide pH range (5.0 to 8.0).

The obtained purified proteases enzymes were further investigated for some factors affecting their activates. Interestingly, the purification steps of protease enzyme produced by *Bacillus brevis*, I-4 resulted in having one protease enzyme namely SHW protease in case of DSHW medium with specific activity of (105436.36 Units/mg. protein/ml) corresponding to purification folds of (16.83) times of the origin.

Similarly, purification steps of protease enzyme produced by *Bacillus brevis var. gelatinoamylolyticus*,I-2 grown on DFW medium resulted in having one protease namely (FW) protease with specific activity of (75677.5 Units/mg. Protein/ml) corresponding to purification folds of (14.45) times of the origin.

Finally, purification steps of protease enzyme produced by *Bacillus brevis var. gelatinoamylolyticus*,I-2 grown on DPW medium resulted in having one protease namely PW protease with specific activity of (67602.56 Units/mg. Protein/ml) corresponding to purification folds of (14.16) times of the origin.

Thus, purification procedures resulted in having three purified enzymes preparations , one of them was produced by *Bacillusbrevis*,I-4 and the other two were produced by *Bacillus brevis var. gelatinoamylolyticus*, I-2.

In all cases, there was a general behavior of the investigated three purified proteases enzymes especially with the factors of enzyme concentration. Since the enzyme activity increased with the increase of enzyme concentration in the reaction mixtures up to certain limit beyond which no increase in enzyme activity could be detected. This behavior however was in accordance with the observation of Bayoumi and Bahobil (16);Bayoumi *et al*.,(17); Bahobil *et al*., (13).

Submerged and solid state fermentation can be used for alkaline proteases production. Media composition (95;96) mainly carbon and nitrogen source (53) and parameters like temperature, pH, agitation speed (39) influence the enzyme production. Each microbe producing alkaline protease require different conditions and medium.

The enzyme activity gradually increased, as the substrate concentration increased for both the crude and the partially purified enzymes and at higher concentrations, there was a gradual decrease in the activity, which may be due to substrate level inhibition of the enzymes. Interestingly, it was found that the optimal gelatin concentration in the reaction mixture of the purified enzymes under investigation was found to be 0.1 % (w/v), this is similar to Bayoumi and Bahobil (16);Bayoumi *et al*.,(17); Bahobil *et al*., (13).

The optimal incubation temperature of the purified enzymes was found to be 55°C. Beena *et al*. (18) explained the reduction in protease activity when exposed to high temperature by its probable thermal denaturation. In addition, Habib *et al.* (38) reported that *Halobacterium* sp. produced protease with lower yield at 50°C. The overall results demonstrated that the strain under study had good enzyme activity between 65 °C and 85 °C. Therefore, it can be classified as athermophilic-protease, data are in agreement with Asker *et al*. (2013).Therefore, results provide a promising enzyme that can be used in detergent industries using hot and/or cold wash cycles, and in other different biotechnological applications.

Mathew and Gunathilaka (57) produced, purified and characterized thermostable alkaline serine protease from *Bacillus licheniformis* NMS-1 and the optimum pH and temperature of this protease were pH 9 and 60°C respectively.

Proteolytic *Bacilli* have been reported to produce two different types of extracellular proteases. Neutral/ metalloprotease with optimal activity at pH 7.0 and alkaline protease with optimal pH range between 9 and 11. The protease enzyme produced by *Bacillus cereus* FT1 showed highest protease activity at a pH range of 9 to 9.5. Although the fact that the three purified enzymes were capable of working within the pH range 5.7-8 using phosphate buffer (0.2M), the optimal pH values of all the purified proteases were found to be (7.0) in the natural point. The optimum activity at alkaline pH ranges suggests that the enzyme is an alkaline protease. A drastic decline in the activity was observed in acidic range. Similarly, the optimum pH for protease activity from *Bacillus* sp. was determined at pH 7.0 as reported by Sevinc and Demirkan (83) who mentioned that it could be a neutral protease. Likewise, the enzymatic activity of different *Bacillus* spp., such as *B. subtilis*ITBCCB 148, *B. subtilis* HS08 and *B. subtilis* S17110wasoptimumat pH 7.5 (35). However, pH 8.0 was the optimum for the enzyme activity of *B. cereus* KCTC 3674, thermophilic *B. cereus*SMIA2 and *B. cereus* BG1 (31). In contrast, Beena *et al*. (18), Asker *et al*. (10) and Habib *et al*. (38) reported alkaline proteases activity from some *Bacillus* spp.

and *Halobaterium* sp. between pH 6-9 with gradual increase in their activity. In all cases, there was a general behavior of the investigated three purified proteases towards , the incubation period of the reaction mixture, since the maximum enzyme activity was obtained was obtained after (60) hours. Generally, commercial proteases derived from microorganisms have optimum activity in the alkaline pH range of 8-12. The pH of laundry detergent falls in the range of 9-12. Different *Bacillus* species are reported to produce alkaline proteases with optimum activity within this range. *Bacillus subtilis* VSG-4 produced an alkaline protease with maximum activity at pH 9. The enzyme activity declined at acidic pH ranges. At non-suitable pH conditions, modification of three-dimensional structures of proteins occurs together with the ionization state alteration of amino acids in the enzyme active site resulting in the loss of enzyme activity. The enzyme with high stability and activity at alkaline pH ranges are most suitable for detergent industry and for cleaning the ultra-filtration membranes.

The optimum temperature for the proteolytic activities of the crude enzyme extracts, prepared from RS and RS1 isolates, ranged from 40ºC for strain RS1 to 70ºC for strain RS at 60ºC (Figure 2a). The optimum pH for the proteolytic activities of RS and RS1 isolates was 7.5 to 8.0 for RS and 7.5 to 8.5 for RS1 (Figure 2b), which is similar to that of other proteases produced from *Bacillus* sp. and *B. stearothermophilus*F1 (40). Wan *et al*. (102) reported thermostability of protease up to60ºC. However, the optimum pH of the RS1 isolate was 8.5, which was slightly higher than the pH 7.0 shown by the zinc proteinase of *A. hydrophila* (55). Viana Daniela de *et al*. (99) also showed that protease activity maximum at pH 7.0. Earlier Prakasham *et al*. (72;73) reported production of protease from *Bacillus* sp. Neutral proteases have been previous reported in *Pseudomonas* sp. and *Burkholderia* sp. *Bacillus cepacia* produces a protease with an optimal pH of 6.0 (58) and a *Pseudomonas fluorescens* strains produce proteases active at neutral pH (60).

On studying the effect of some activators and/ or inhibitors on the activity of the three purified proteases enzymes, five substances in different five concentrations were applied (100,200,300,400 and 500 ppm). It was found that the maximum SHW protease activity was obtained in the presence of calcium chloride at concentration 200-300 ppm and magnesium sulphate at concentration 200 ppm , while the achieved in the presence of calcium chloride and magnesium sulphate at concentration 200 ppm. Whereas, cadmium chloride, lead chloride and nickel sulphate showed an inhibitory effect on the activity of the three purified protease enzymes.

Purified protease samples were assayed in the presence of inhibitors. The failure of either serine or cysteine proteinase inhibitors PMSF suggest that RS1 protease is a metalloproteinase(71). The strongest inhibitor of protease activity was 1-10-phenanthroline, which is the preferred metalloproteinase inhibitor (Powers and Harper,1988).Proteases are one of the most important groups of both industrially and academically important enzymes accounting for around 65% of annual enzyme market (72;73). They have a history of applications in food and detergent industries where the alkaline proteases hold the biggest share of the enzyme market worldwide (36). Alkaline protease use as ingredient in detergents is largest application of this enzyme. They also have applications in leather industry, medical diagnostics, and recovery of silver from X-rays, food and feed industry etc. Due to their widespread applications, many industries have started its production at commercial level.

The valuable applications of industrial utilization of enzymes had led to the development of a specialized area of enzyme engineering. In our investigation, the three crude protease enzymes were investigated for their ability to be used in leather bating industry, where, the wool of the hides and skins of the animals was removed, and then treated with enzymes and chemicals to make them soft and supple and to prepare them of tanning. Various researchers characterized alkaline proteases from different sources to use them for specific purposes. Alkaline proteases due to its high pH range and high thermo stability have many applications in detergent and leather industry.

Proteases are one of the standard ingredients of all kind of detergents ranging from those used for household laundering to the reagents used for cleaning contact lenses or dentures. The enzymes must be stable at high temperature and pH and have compatibility with bleaches, surfactants, chelating and oxidizing agents present in the detergents (66). Alkaline proteases from *Bacillus mojavensis*(19), *Bacillus sp*. L21 (91) and *Bacillus licheniformis* RP1 (82) have also been reported useful for detergent industry.

A good detergent additive protease enzyme must be stable and compatible with the compounds present in

the commonly used detergent such as surfactants, bleaches and oxidizing agents. Alkaline proteases greatly contributed to the improvement and development of industrial detergents. These detergents are effective at any pH and temperature condition that are helpful in industrial cleaning. Enzymes that are used in laundry industry include proteases, lipases, cellulases and amylases (44; 3). These proteases help in the removal of any stain like blood, egg, gravy even in high pH conditions (80; 9). Biotechnologists faced many problems regarding application of proteases; one major of them is compatibility issue with various other detergents. Recently, alkaline proteases from *Bacillus cereus*, *Bacillus pumilus* strain CBS, *Streptomyces sp*. strain AB1, *Bacillus licheniformis*, *Aspergillus flavus*, *Aspergillus niger*, *Bacillus brevis*, *Bacillus subtilis* AG-1 have exhibited excellent detergent compatibility in the presence of certain stabilizers such CaCl2 and glycine (1;88). Subtilisins related to thermo stability and resistance to chelators, survive to extremes of high alkalinity and chelator concentration (30). Alkaliphilic *Bacillus* strains have been used for the isolation of oxidatively stable serine protease to prevent loss of activity when used in detergents.

In our investigation, the three purified proteases were applied separately in laundry detergents, where the three purified proteases enzymes were compared with a traditional detergent OMO. These gave very good results in comparison with OMO detergent. Proteases are used in the soaking, dehairing and bating stages of preparing skins and hides. The enzymatic treatment destroys undesirable pigments, increases the skin area and thereby clean hide is produced. Bating is traditionally an enzymatic process involving pancreatic proteases. Varela *et al.,* (95;96) and Jain *et al*.,(45) reported the use of *Bacillus subtilis* IIQDB32 alkaline protease for unhearing sheep's skin.

Conclusion

It may be concluded that *B.brevis*, I-4 and *B.brevis var. gelatinoamylolyticus*, I-2 successfully utilized industrial wastes for proteases production under thermostable conditions. In addition, the obtained data of the application of the present thermostable neutral proteases in the fields of biodetergents and leather bating industries refers to a great hope in the course of applying the present proteases in such industrial purpose, especially if their production was performed in large scale of SSF conditions.

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