Production, Purification and Characterization of Alkaline and Thermostable Protease by *Shewanella putrefaciens*-EGKSA21 Isolated from El-Khorma Governorate KSA

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Abstract: Proteases catalyzes the hydrolysis of protein. Among the various proteases, bacterial proteases are the most significant when compared with animal and fungal proteases. The aim of the present study was to production of alkaline thermostable bacterial protease for application in biodetergent technology. Screening studies were carried out for twenty one thermophilic bacterial isolates with respect to their ability to produce both protease and lipase when grown on mineral salts medium supplemented with gelatin as only source and carbon and energy at 50°C and pH 9. The most potent thermophilic bacterial isolate for production of two enzymes was identified as *Shewanella putrefaciens*-EGKSA21. The optimum incubation temperature and pH for maximum alkaline–thermostable protease production were 50°C and 9 under fermentation conditions. Optimum substrate concentrations for protease production were 3 % gelatine. The best carbon sources that induce protease production by *Shewanella putrefaciens*-EGKSA21 was D (+) arabinose and D-xylose. Potassium nitrate, sodium nitrate and ammonium chloride were the optimal nitrogen sources for alkaline–thermostable protease production by *Shewanella putrefaciens*-EGKSA21. Maximum protease production was observed at the end of 48hrs. The overall steps protocol resulted in raising the purification fold to 411.9 times. Optimum incubation temperature and thermal-stability were 50 and 50-55 ºC for the purified protease. The activities of the purified enzymes increased gradually with the increase of time up to 48 h incubation of the reaction mixture. The activities of the purified enzyme increased gradually by the increase of enzyme concentrations. The effect of different metalic ions on the purified enzyme activities recorded that Sodium azide (50 ppm), Lead acetate (50 ppm) and EDTA (50ppm) exhibited maximal activities while cadmium chloride and magnesium chloride inhibited the purified enzyme activities. The purified enzymes exhibited good stability towards organic solvents. The crude and purified protease of produced by *Shewanella putrefaciens*-EGKSA21 bacterial strain with a potential to be a candidate for the application in the detergent industry.

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1. Introduction

Proteases constitute one of the most important groups of industrial enzymes and have applications in different industries such as detergent, food, feed, pharmaceutical, leather, silk and for recovery of silver from used x-ray films. Proteases are one of the most important classes of biocatalysts from an industrial point of view, occupying a major share of 60% of the total enzyme market (Pandey, 2003). These biocatalysts hydrolyze peptide bonds in proteins and hence are classified as hydrolases and categorized in the subclass peptide hydrolases or peptidases (Ellaiah *et al.*, 2002). Because of this functional property, they are widely used in laundry detergents, leather processing, protein recovery or solubilization, meat tenderization, and the biscuit and cracker industries (Johnvesly and Naik, 2001). However, other application potentials of these enzymes depend on the nature of catalytic activity with respect to reactant medium, which led to the classification of proteases as acidic, neutral, and alkaline. Among these different biocatalysts, alkaline proteases have wide application spectra and novel properties due to their exotic catalytic nature. Hence, these proteases and their producing organisms attracted attention of scientific community to understand the protein chemistry and protein engineering to enhance their utilization niche (Germano *et al*., 2003).

Though several microorganisms such as bacteria, fungi, yeast, plant, and mammalian tissues are known to produce alkaline proteases (Ellaiah *et al*., 2002; Prakasham *et al*., 2005 a&b), with increasing industrial demand for proteases it is expected that hyperactive strains will emerge and that the enzymes produced by new exotic microbial strains could be used as biocatalysts in the presently growing biotechnological era. Available literature information indicates that, among all protease-producing microbial organisms, the *Bacillus* genus assumes importance because of its potential for production in large amounts

(Kumar *et al*., 1999; Mabrouk *et al*., 1999; Mehrotra *et al*., 1999). Moreover, several medium components such as nitrogen and carbon sources, physiological factors such as pH, incubation temperature and incubation time, and biological factors such as the genetic nature of the organism influences the metabolic/biochemical behavior of the microbial strain and subsequent metabolite production pattern (Kumar *et al*.,1999; Ellaiah *et al*., 2002; Prakasham *et al*.,2005 a&b). Hence, in commercial practice, the optimization of medium composition is one of the essential steps to maintain a balance between the various medium components to minimize the amount of unutilized components at the end of fermentation and have cost-effective metabolite yield (Kumar *et al*., 1999; Prakasham *et al*., 2005a&b).

In general, no defined medium has been established for the best production of any metabolite because the genetic diversity present in different microbial sources causes each organism or strain to have its own special conditions for maximum product production (Ellaiah *et al*., 2002). Therefore, it is essential to have a detailed investigation on newly isolated microbial strain for production pattern under different environmental conditions and in an optimized pattern to achieve maximum production benefit. For effective triggering of alkaline protease production, it is highly imperative to optimize all fermentation conditions including medium composition, which further facilitates economic design of the full-scale operation system. However, it is impractical to optimize all parameters and to establish the best possible conditions by interrelating all parameters, as this involves numerous experiments to be carried out with all possible combinations (Prakasham *et al*., 2005 a&b; Sreenivas Rao *et al*., 2004). Experimental design based on statistical tools is known to provide economic and practical solutions in such cases.

Optimization procedures developed to optimize the biotechnological processes consist of an empirical modeling system developed on a full factorial central composite technique for evaluation of the relationship among the parameters that influence the production process. The Taguchi method of statistical procedure is mainly based on orthogonal arrays to provide a systematic, simple, and efficient approach (Phadke and Dehnad, 1988). It allows a more realistic arrangement of the experimental sets working with the understanding system, parameter, and tolerance designs (Phadke and Dehnad, 1988). Importance of this procedure has been evaluated in several microbial secondary metabolite production processes (Prakasham *et al*., 2005 a&b; Sreenivas *et al*., 2004).

Alkaline proteases assume significant importance in laundry, food, leather and silk industries (Priest, 1977; Turk, 2006; Subba, 2008 a &b).However, novel proteases with high activity profile at versatile environments have major application potential in pharma, diagnostic, detergent, tannery, amino acid production, contact-lens cleaning agents, effluent treatment, enzymic debridement and support the natural healing process in the skin ulcerations (Subba, 2009).

Proteases properties like physical, biochemical, thermal, molecular and catalytic, vary with the genetic nature of the producing organism (Geok *et al*., 2003). Each enzyme is specific and their use depends on stability and robustness against solvents, surfactants and oxidants.Hence, basic catalytic knowledge is one of the pre-requisites for evaluation of its potential for biotechnological application (Prakasham *et al*., 2006; Subba *et al*., 2008a&b).

This study aims (i) isolation of thermophilic and alkaliphilic bacterial isolates from local region in KSA (ii) Production of the thermoalkaliphilic proteases from the potent bacterial isolate under investigated optimal nutritional and environmental conditions and (iii) characterization of the purified enzyme.

2. Materials and Methods

Construction of standard curves

A stock enzyme preparation:

A stock solution of (50,000 µg/ml) purified protease enzyme supplied by Sigma chemicals Co. was prepared in Tris– buffer (0.2 M) at pH 9.0. Descending dilutions were prepared.

Construction of the standard curves:

After preparing the required dilutions for protease enzyme, only 0.1 ml of each dilution was transferred to each well in the assay plate. Three wells were used for each dilution. Incubation was performed at 55ºC for 18 h. Then mean diameters of clearing zones (mm) were determined for protease concentration. A standard curve was constructed relating protease concentrations applied against their corresponding mean diameters of clearing zones (mm).

The obtained standard curves were used for estimating the protease activities in terms of µg/ml and then units (U). One unit is defined as the amount of enzyme protein (mg) required to exert one unit of clearing zone (mm) in one unit of time under all the specified conditions of protease assay (clearing zone technique).

Protein determination:-

Protein of protease preparations was determined by the method of Lowry *et al.* (1951) using Bovine Serum Albumin as standard.

Growth and maintenance medium:

Nutrient agar (NA) medium:

This medium contained (g/l): Peptone, 5; sodium chloride, 5; beef extract, 3; agar-agar, 15; and distilled water up to 1000 ml. The ingredients were dissolved by heating, pH was adjusted at 7, and sterilized at 121ºC for 15 min (Shiriling and Gottlieb, 1966).

Production media:

The basal medium (BM) was prepared according to Vincent (1970). It contained the following (g/l): Sucrose, 10; KNO_3, 0.6; KH_2PO_4, 1; $MgSO_4$, 0.25; and $CaCl_2$, 0.1 was found most convenient for the production of different enzymes. It was modified to include the following constituents: (g/l) NaCl, 6; $(NH_4)_2SO_4$, 1; yeast extract, 1; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.1; $CaCl_2$, 0.1, and distilled water up to one litre.

Isolation of thermophilic bacteria:-

Thermophilic bacterial isolates were isolated from different localities alkaline desert soil samples collected from different localities of El-Khorma governorate, Taif, Kingdom of Saudi Arabia (KSA). Soil samples were applied using the soil dilution plate technique.

Qualitative screening test media, methods and conditions (First survey):

Proteolytic enzyme production:

The same ingredient of BM was used in addition to 1% gelatin and 1.5% agar and was adjusted at pH 9. It was autoclaved at 1.5 atm. for 15 minutes. Plates of the same size were poured with equally amounts of agar medium in each Petri dish. After cooling, each plate was inoculated in the center with bacterial isolate onto the surface, then incubated at 55ºC for 24 h. Bacterial growth and clearing zones of the medium after addition of acidic mercuric chloride solution were investigated and taken as criteria for determining the proteolytic activity.

Media used in screening test for selecting the most potent bacterial isolates:

For screening protease(s) activity, the BM was used and a gelatin waste was used as a substrate by a concentration of 5%. Enzyme activity was detected by gelatin clearing zone (GCZ) technique.

Assay media:

This medium was prepared according to gelatin clearing zone (GCZ) technique according to Ammar *et al*., (1991). The assay plates containing 1% (w/v) gelatin and 1.5% (w/v) agar for solidification, to be dissolved up to 100 ml of Tris-buffer (pH 9).. At the end of incubation period, protease(s) activity was detected by flooding each plate with 10 ml freshly prepared acide mercuric chloride solution (Barrow and Feltham, 1993). Mean diameters of clearing zones were recorded, calculated and then taken as indication for proteolytic activities.

Identification of the most potent thermophilic bacterial isolates:-

The most potent bacterial isolate EGKSA21 was identified by examination of their morphological physiological and biochemical characteristics.

Reagent:

Acid mercuric chloride reagent was prepared as the following: Mercuric chloride, 12g; distilled water, 80 ml; and conc. HC1 16 ml. $HgCl_2$ mixed with water, the acid added, and the mixture mixed well until solution was completely prepared(Barrow and Feltham, 1993).

Preparation of cell-free-filtrate (CFF) from the thermostable protease production medium:

This was performed by preparing the previously mentioned production medium under all investigated optimal nutritional and cultural conditions. At the end of incubation period, the bacterial growth was harvested by centrifugation at 5,000 r.p.m. for 20 min. The supernatant was obtained and preserved into the refrigerator as a crude enzyme and/or assayed at the same times.

Parameters controlling the thermostable protease productivity:

The effect of different temperatures; pH values; substrate concentrations; carbon and nitrogen sources and different incubation periods on protease production by EGKSA21 were determined by growth of organisms in fermentation medium. Protease productivity was measured through assay as previously mentioned.

Purification of protease enzyme:

The following steps were performed during the course of production, and purification of thermostable protease. *Shewanella putrefaciens*-EGKSA21 was allowed to grow under the optimal fermentation conditions for protease production.

At the end of incubation period, the bacterial growth was harvested by centrifugation at 5,000 rpm for 15 min. The supernatant was filtrated, and the obtained cell-free filtrate was preserved in the refrigerator as a crude enzyme according to Ammar *et al*. (1985).

Ammonium sulphate fractionation:

The chart of Gomori (1955) as mentioned by Dixon and Webb (1964) was applied to calculate the solid ammonium sulphate to be added to achieve any given concentration of the cell free filtrate under investigation.

Applying on column chromatography technique on sephadex G 200 :

The dialyzed-partially purified-protease preparation was applied onto a column packed with sephadex G 200. This was equilibrated with Tris-buffer (0.2 M) adjusted at pH 9, then eluted with the same buffer. Preparation of the gel column and the fractionation procedure was carried out as mentioned by Soliman (2003).

Factors affecting the purified thermostable enzymes activities:

Effect of incubation temperatures:

This was carried out by incubation of purified protease after pouring in assay medium at different incubation temperatures viz. 35, 40, 50, 55, 60, 65 and 70ºC respectively.

Effect of temperature stability:

This experiment was designed to determine the range of temperature within which the enzymes maintained their activities. The experiment was carried out by incubating the purified enzymes for 2 h at different temperatures viz., 50, 55, 60, 65, 70, 75, 80, 85 and 90ºC, respectively. At the end of incubation temperature, the replicate tubes were cooled and assayed for each purified enzyme to determine the retained enzyme activity as previously mentioned.

Different pH values:

The purified enzyme was incubated at different pH values viz., 6, 7, 8, 9, 9.5, 10 and 11 by using phosphate, Tris- or glycine buffers. After sterilization, pouring and solidification of the assay plates, three wells were made in each plate and then 0.1 of ml purified enzymes were inoculated in the well. Then the plates were incubated at 55ºC for 18 h. The protease activity was determined as previously mentioned.

Metallic ions as enzyme activators and /or inhibitors:

In this experiment, the purified enzymes were supplemented with different separated metallic ions in the form of cadmium chloride, magnesium chloride, sodium azide, lead acetate and EDTA. Different metallic ions concentrations were applied viz., 50, 100, 250, 500 and 1000 ppm for each metallic ion. Enzymes activities were assayed as previously mentioned.

Stability of the purified enzymes in the presence of organic solvents:

The aim of this experiment is to study the computability of the purified protease with organic solvent.

Amino acid analytical data of the purified enzyme

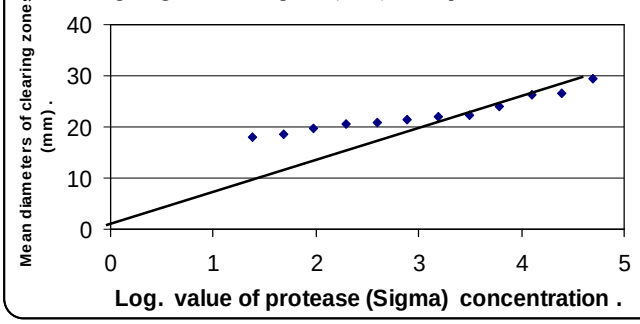
The hydrolyzed protein amino acids have been determined in the central Lab. for Food, Agricultural Research Center according to the methods described by Pellet and Young (1980). LKB Alpha plus high performance Amino Acid Analyzer LKB Biochrom. LTD England was used for this purpose. Retention time and area was determined using Hewlett Pakard 3390 recording integrator. The concentration of each amino acid GM/16 G.M., nitrogens was calculated by special designed program.

3. Results:

Construction of standard curve for determination of enzyme activity:

The productivity of protease was estimated in term of mean diameter (MD) of clearing zones (mm) using a special standard curves, prepared for such a purpose and covering the range of (24.4–50 000 µg protein /ml) as shown in figure (1). The standard protease (Sigma) was used by using GCZ plate techniques. Mean diameters of clearing zones in term of (mm) and their corresponding log. values of enzyme concentrations were recorded.

Fig.(1):The relation of Log. Value of standard protease concentrations to their corresponding mean diameters of clearing zone (mm) using the gelatin clearing zone(GCZ) technique..



Qualitative screening test for selection of the most potent thermostable protease producers:

Twenty one thermophilic bacterial isolates were isolated from different localities of different alkaline soil samples collected from khorma governorate , Taif, Kingdom of Saudi Arabia (KSA) . These isolates were purified, and subjected to a screening program in order to evaluate their proteolytic and lipolytic activities by measuring (observing) the hydrolysis of gelatin and tributyrin around the bacterial colonies. Out of twenty one isolates there was found that thirteen bacterial isolate gave proteolytic

productivity and isolate number 21 was found to be the best protease producer while eighteen isolates considered to have a good lipolytic activity and isolate number 21 also was found to be the best lipase producer (Table 1).

Quantitative screening for selection of the most potent protease producer thermophilic bacterial isolates:

Data recorded in table (2) showed that bacterial isolate number 21 gave a higher protease productivity by gelatin clearing zone quantitatively. Bacterial isolate number 21 gave the highest proteolytic activities where it reached up to 25 (mm) compared to other isolates. From the previous results concerning the qualitative and quantitative screening of two enzymes qualitatively and protease quantitatively production, bacterial isolate number 21 were selected for their ability to produce the two enzymes in high enzyme production. The most potent thermophilic bacterial isolate was subjected to identification.

Identification of the two most potent bacterial isolates:

The most potent bacterial isolate 21 was subjected to an identification program to the species level.

Table (1): A screening test for the selection of the most potent two hydrolytic thermostable enzymes producers out of twenty one pure thermophilic bacterial isolates

Bacterial isolate code no.	Bacterial Code No.	Extracellular thermostable hydrolytic enzymes production	
		Protease(s)	Lipase
1	15	0	22
2	18	0	26.5
3	20	18	17
4	21	42	28
5	22	34	26
6	24	31	18.5
7	41	0	18
8	42	26	21.5
9	50	38	16.5
10	111	0	19.5
11	112	29	22
12	121	25	16
13	122	32	22
14	131	0	0
15	132	0	0
16	191	0	25
17	212	29	22
18	221	40	20
19	2221	32	17
20	2222	29.5	17
21	SH1	0	0

Table (2): Relation of thermostable protease production by most potent bacterial isolates at 55ºC for 48h. using GCZ techniques

Bacterial isolate code no.	Bacterial Code No.	Extracellular thermostable protease production
1	20	19.5
2	21	25
3	22	18.5
4	24	18
5	42	18
6	50	20
7	121	19
8	122	18
9	212	18.5
10	221	21
11	2221	20
12	2222	12

General characteristics of the most potent thermophilic bacterial isolates :

All morphological characteristics and stain reaction led to the fact that the bacterial isolate under identification are suggestive of being belonging to the genus *Shewanella* , Gram-negative aerobes to facultative anaerobes, non Endo-spore formers. Amylase, cellulase, protease, lipase and catalase positive while, urease and caseinase negative. The cells were able to grow between (30-60°C) temperature interval. The cells were able to grow in the presence of (0-2%) of NaCl at pH 9.0

Specific characteristics of the most potent thermophilic bacterial isolate:

This isolate appears waxy in colour on nutrient agar medium, rod shaped, nitrate reduced, assimilate of N-acetyl –glucosamine and malic acid. Produced cyctochrome oxidase. *Shewanella* was able to grow facultative anaerobically, it is suggested to belong to the species putrefaciens. It could be given the tentative name *Shewanella putrefaciens*.

Parameters controlling the four thermostable enzymes production

Data recorded in table (3) showed a summary of the optimal nutritional and environmental conditions for thermostable hydrolytic protease production by ***Shewanella putrefaciens*-EGKSA21**.

Purification of protease produced by *Shewanella putrefaciens*-EGKSA21 allowed to grow on BM under fermentation conditions.

In this section enzyme produced by ***Shewanella putrefaciens*-EGKSA21** previously grown on BM supplemented with gelatin as a preferable substrate supplemented with mineral salts under the optimum nutritional and environmental conditions recorded in table (3) were purified to homogeneity as previously mentioned by performing ammonium sulphate fractionation, dialysis, and applying column chromatography on sephadex G200. The obtained purified enzymes were further investigated for some factors affecting its activity.

Table (3): A summary of the optimal nutritional and environmental parameters controlling four thermostable hydrolytic enzymes productivities by *Shewanella putrefaciens*-EGKSA21 under solid or semi solid fermentation conditions.

No	Parameter	*Shewanella putrefaciens*-EGKSA21
		Protease(s)
1	Temperature(ºC)	50
2	pH value	9
3	Substrate concentration	3%
4	Carbon source	L(+) Arabinose
5	Nitrogen source	Potassium nitrate
6	Inoculme size (ml)	1 ml
7	Incubation period(hours)	48h

Purification of thermostable protease:-

The following steps were performed during the course of the purification of enzyme under study. where protease was produced by *Shewanella putrefaciens*-EGKSA21 allowed to grow on BM supplemented with gelatin under submerged fermentation condition at 50ºC under all the optimal conditions.

Enzyme production and preparation of CFF :-

The most potent bacterial strain was allowed to grow on the BM supplemented with gelatin under all the previously mentioned optimal submerged fermentation conditions as shown in table (3) for production of protease by *Shewanella putrefaciens*-EGKSA21. At the end of incubation period, 1000 ml of protease production medium was extracted and collected separately. Centrifugation of the obtained extracts was done at 5000 rpm for 15 min at 10ºC. The precipitate was collected and tested for determination of protease activity and protein content and corresponding specific activity was calculated.

Fractional precipitation by ammonium sulphate:

Results recorded in table (4) indicated that the most active enzyme protein preparation was obtained at an ammonium sulphate level at 60-80 % for protease was at 80%, where specific activity was 21658.5 U/mg ^{1} proteins. Only 42 ml were obtained at the end of the process of dialysation against tap water of protease.

Concentration by dialysation against sucrose:-

The most active ammonium sulphate fractions previously obtained at the best saturation, (50 ml) protease was dialyzed against distilled water followed by dialysis against sucrose crystals until a volume of 4.5 ml was obtained (Table 4).

Table (4): Ammonium sulphate precipitation pattern of the protease produced by *Shewanella putrefaciens*-EGKSA21 allowed to grow on BM supplemented with gelatin at 50ºC.

$(NH_4)_2SO_4$ saturation level (%)	Protease activity (U/ml) (x)	Protein content (mg/ml) (y)	Specific activity (U/mg^{-1}protein) (x/y)	Purification fold
CFF	1304.936425	0.48	2718.617557	1.0
20	1867.96537	0.622	3003.159759	1.1
40	3827.61067	0.88	4349.557	1.59
60	5479.07473	1.3	4214.6728	1.55
80	**7843.0868**	**1.2**	**6535.9007**	**2.4**
100	911.611698	0.82	1111.72157	0.4

Preparation of sephadex G-200 gel filtrate column and applying the enzyme sample:

Data recorded in table (5) showed that, there were one active peaks. It was found that the first beak (fractions 10-18) has the highest specificity and the fraction number 11 was reached to the maximum specific activity up to 5095.221 U/mg^{-1} protein.

Table(5): Fractionation pattern of protease produced by *Shewanella putrefaciens*-EGKSA21 at 50 ºC using sephadex G-200 column chromatography technique.

Fraction No.	Protease activity (U/ml) (x)	Protein content (mg/ml) (y)	Specific activity (U/mg^{-1}protein) (x/y)
1-9	UD	UD	UD
10	217.116	0.0236	9199.830
11	1936.184	0.380	5095.221
12	1618.292	0.353	4584.398
13	1304.946	0.428	3048.9392
14	684.205	0.477	1434.6868
15	848.504	0.396	2142.6868
16	761,938	0.179	4256.6368
17	761.938	0.112	6803.0178
18	571.869	0.123	4639.34114
19-40	UD	UD	UD

Characterization of the purified protease produced by *Shewanella putrefaciens*-EGKSA21 at 50^{O}C.

The aim of the present series of experiments was to investigate some properties of the partially purified enzyme produced by ***Shewanella putrefaciens*-EGKSA21** allowed to grow on BM and incubated under all optimal nutritional and environmental submerged fermentation conditions. These properties include:- Effect of incubation temperature, pH values, pH stability, inhibitors and/or activator and stability with organic solvent on hydrolytic thermostable purified protease activity.

1- Effect of incubation temperature:

Results recorded in table (6) showed that the maximum activity of thermostable protease was obtained at 55ºC, where it reached up to 1867.96537 U/ml.

Table (6): Different incubation temperatures in relation to the activity of purified protease produced by *Shewanella putrefaciens*-EGKSA21 .

Incubation temperature (ºC)	Protease activity (U/ml)
35	715.092
40	1304.946
50	3199.175
55	1867.96537
60-70	0

2-Effect of different pH values on the activity of purified enzymes:

Data recorded in table (7) emphasized that the best pH value that fulfill the highest protease activity was 9 where it reached up to 5479.074 U/ml.

Table (7): Different pH values in relation to the activity of the purified protease produced by *Shewanella putrefaciens*-EGKSA21.

pH	Protease activity (U/ml)
Control	2673.919
7	74.02077
8	2673.919
8.5	3827.610
9	5479.074
9.5	3827.610
10	217.116
11	74.0207

3- Effect of metallic ions (activators and / or inhibitors) on the purified protease activities:

Results shown in table (8) that nearly all of the tested metallic ions exhibited inhibition effect on the purified protease activity.

4-Stability of the purified enzymes in the presence of organic solvents:

The aim of this experiment is to study the computability of the purified enzyme with organic solvent. This is an important observation because of the fact that enzyme incompatibility with organic solvent is the reason for its stability. Data recorded in table (9) showed that 40% acetone concentration did not exert any inhibition for protease activity.

Table (8): Different inhibitors and/or activator in relation to the activity of the purified protease produced by *Shewanella putrefaciens* EGKSA21

Activator and /or inhibitor	Concentra-tion (ppm)	Protease activity (U/ml)	Inhibition (%)
Control	**0.0**	**32930.97301**	**0.0**
Cadmium chloride	50	322.1435	99.02
	100	0	0
	250	0	0
	500	0	0
	1000	0	0
Magnesium chloride	50	241.78305	99.265
	100	0	0
	250	0	0
	500	0	0
	1000	0	0
Sodium azide	50	3199.1751	90.285
	100	2872.7919	91.276
	250	2673.919	91.880
	500	2488.8137	92.442
	1000	2234.9023	93.213
Lead acetate	50	3827.6106	88.376
	100	3562.639	89.181
	250	2234.9023	93.213
	500	1936.1846	94.120
	1000	1677.3935	94.906
EDTA	50	3562.639	89.181
	100	2977.708	90.957
	250	2579.706	92.166
	500	1936.18	94.120
	1000	1304.946	96.037

5- Amino acids analysis of the purified enzymes:

Data recorded in table (10) showed that, 12 amino acids were detected in addition to ammonia. Alanine and glutamic acid represented the highest value i.e 305.28 and 180.3 µg/ml respectively.

Table (9): Stability of the purified protease produced by *Shewanella putrefaciens* EGKSA21in the presence of acetone.

Acetone concentration (%)	Protease activity	Protein content	Volume(ml)
20	8129.51	0.312	10
40	21412.590	0781	15
60	11227.063	1.4	10
80	5479.074	1.1	5
Before precipitation	23005.1513	0.821	9.5
After precipitation	13432.470	1.2	40

Table(10):A summary of amino acids analytical data of *Shewanella putrefaciens*-EGKSA21 purified protease.

No.	R.T	Amino acid	(µg/ml).	(µg/ml).X4
1	11.62	**Aspartic**	67.17	268.68
2	14.75	**Threionine**	20.59	82.36
3	16.17	**Serine**	39.70	158.8
4	**18.27**	**Glutamic acid**	**180.3**	**721.2**
5	27.03	**Alanine**	305.28	1221.12
6	32.62	**Valine**	48.94	195.76
7	35.45	**Methionine**	7.02	28.08
8	38.47	**Leucine**	20.47	81.88
9	42.93	**Phenylalanine**	67.94	271.76
10	50.97	**Histidine**	6.23	24.92
11	53.85	**Lysine**	70.09	280.36
12	62.75	**Arginine**	90.77	363.08

R.T.: Retention time

4. Discussion

Enzyme production is a growing field of biotechnology and the world market for enzyme is over $1.5 billion and it is anticipated to double by the year 2008 (Lowe, 2002). The main object of the present work was an investigation of screening, production, purification and characterization of thermo-alkalostable enzymes for application in detergent technology has been undertaken.

In this regard twenty one bacterial isolates were isolated from different soil and water samples collected from different localities in Khorma Governorate, Taif, Kingdom of Saudia Arabia (KSA). These bacterial isolates were grown at 50°C and at pH 9 to be able to produce a thermostable and alkalophilic enzymes which favorable to be used as additive to bio-detergent formulations(Bayoumi *et al*., 2011; Bayoumi and Bahobil,2011).

A screening test of proteolytic and lipolytic productivities of all bacterial isolates resulted in the fact that, only thirteen and seventeen bacterial isolates gave a good proteolytic and lipolytic productivities respectively.

Shewanella spp. are widespread Gram-negative bacteria that have been isolated from many different habitat. This adaptability is supported by a flexible metallic capability , particularly in the choice of respiratory electron acceptors (Gordon *et al*., 2000).

The present investigation was also aimed at optimization of medium components which have been predicted to play a significant role in enhancing the production of proteases (Gupta *et al*., 2002). Hence proper combination of various cultural conditions can be established in order to *Shewanella putrefaciens*-EGKSA21 for high secretion of alkaline protease. *Shewanella putrefaciens*-EGKSA21 was allowed to grow in media of different pH ranging from 7-11. Maximum enzyme activity was observed in medium of pH which was the optimum pH 9 for *Shewanella putrefaciens*-EGKSA21. The majority of microorganisms producing alkaline proteases show growth and enzyme production under alkaline conditions (Tsujibo *et al*.,1990; Dunaevsky *et al*., 1996).

Enzyme activity recorded at different temperature revealed that the *Shewanella putrefaciens*-EGKSA21 yielded maximum protease production at 50. The temperature was found to influence extracellular enzyme secretion; possibly by changing the physical properties of the cell membrane (Rahman *et al*.,2005).

Various sources of carbon such as D(-) glucose, L(+) arabinose, D-xylose, D(-) lactose, maltose, D(-) mannitol, dextrin, cellulose, starch, inulin , Myo-inositol and D-sorbitol were used to with the original carbon source in growth media . Results obtained were showed that, L(-) arabinose brought the highest protease production compared to other carbon sources at 50ºC,pH 9 and 48 h of incubation. For commercial production , sugars like fructose, lactose, lactose, mannitol , sucrose will be prohibitive due to their cost (Suresh *et al*., 2008).

Production of extracellular protease has been shown to be sensitive to repression by different carbohydrate and nitrogen sources (Levisohn and Aronson,1967; Haulon *et al*., 1982). The effect of nitrogen source was studied in the growth medium , where sodium nitrate, potassium nitrate, ammonium oxalate, ammonium molybedate, peptone, urea ammonium chloride. Among the various nitrogen sources tested potassium nitrate was found to be the nitrogen source for alkaline protease production.

The application in the detergent industry does not require high-purity of enzymes and generally require use of the crude or partially purified enzyme preparation. However, it is significant to obtain enzymes with higher specific activity for their kinetic characterization. Since *Shewanella putrefaciens*-EGKSA21 proved to be the most potent proteolytic and lipolytic bacterial strain, they were selected for the

purpose of production, purification and investigating properties of proteases biosynthesized by this particular strain. However, there was no universal prescription for various techniques which could be applied for enzyme purification (Wilson and Walker, 1994).

Purification process includes essential steps as precipitation of protein using ammonium sulphate or other precipitants as low molecular weight alcohols and gel filtration using different column chromatography. Needless to say, that, most of the enzyme purification schemes described in the literature focused on purifying small amounts of the enzyme to homogeneity to characterize it. Little information has been published on large-scale processes for commercial purification. Most commercial applications of enzymes does not require highly pure enzyme. Excessive purification is expensive and reduces over all recovery of the enzyme. In the present study, the purification procedure included preparation of cell free filtrate; applying precipitation technique, dialysis; and then passing the enzyme preparation through Sephadex G-200 column chromatography techniques.

Fractional precipitation of enzymes was carried out firstly by ammonium sulphate since it is highly soluble in water, cheap and has no deleterious effect on structure of protein, so for all these reasons, precipitation by ammonium sulphate was selected as a first step of purification program. Many investigators used ammonium sulphate precipitation processes (Omar, 2000; Roushdy, 2001; Sodhi *et al.*, 2005).

In a trial to precipitate enzymes by ammonium sulphate, results revealed that, increasing the concentration of ammonium sulphate resulted in an increase in specific activity of amylase up to 80% saturation, a decrease in specific activity was recorded above this value. On the other hand, 80% saturation with ammonium sulphate was proved to be the best concentration for maximal specific activity for protease.

In complete accordance with the present results for protease, Chitte *et al.* (1999) found that, the crude protease enzyme was concentrated by precipitation with 80% saturation of ammonium sulphate. Hutadilok *et al.* (1999) used 80% saturation of ammonium sulphate for protease purification. Uchida *et al.* (2004) recorded that, 75% ammonium sulphate saturation for *B.subtilis* CN2.

Gel filtration of the thermostable protease on sephadex G-200 showed that, the enzyme activity was detected firstly, in fractions 10-18 where the highest specific activity 5095.221 U/mg^{-1} protein was recorded in fraction 11. The major peak was in fraction no. 11.

Moharam *et al.*, (2003) purified protease from two strains of *B.sephaericus* (IS 2362) and NRC 69 by 58 and 126 folds respectively by using ammonium sulphate fractionation and sephadex G-100 column chromatography while, Nilegaonkar *et al.*, (2002) recorded 10 folds increase in specific activity of protease when purified using gel filtration on sephadex G-100. Uchida *et al.*, (2004) purified protease produced by *B.subtilis* CN_2 by 272 folds with an over all yield of 9.2% from the culture supernatant.

In the present study, amino acids analytical data of the purified protease produced by *Shewanella putrefaciens*-EGKSA21 indicated a total number of 15 amino acids with glutamic acid and alanine showing the highest value. Similarly, Mahmoud, (2004) found that, glutamic acid gave the highest value for purified protease produced by *Pseudomonas aeruginosa* B8.

Concerning the purified protease, it was found the temperature and pH optima 60ºC and 10.5 respectively, and the enzyme was thermostable up to 50-55ºC and pH stable at 6 and 10 where the maximal activity was obtained.

Interestingly, in the present work, it was found that, Pb^{+2}, Na^{+} and EDTA stimulated alkaline-thermostable protease activity produced by *Shewanella putrefaciens*-EGKSA21 while Cd^{+2} and Mg^{+2} make inhibition at high concentration (100-1000 ppm). Confirming that, these cations take part in the stabilization of the protease structure and are required for protection against thermal denaturation (Paliwal *et al.*, 1994), and play a vital role in maintaining the active confirmation of the enzyme at high temperatures (Gupta *et al.*, 2002).

Similar results was recorded by Yang *et al.*, (2000) who found that, the activity of purified protease enzyme produced by *B.subtilis* was increased in the presence of Mn^{+2}, Fe^{+2}, Zn^{+2}, Mg^{+2}, Co^{+2} ions, but it was inhibited by Hg^{+2} ions.

5. Conclusion

The aim of this research work was to isolate and identify high protease production from local habitat. *Shewanella putrefaciens*-EGKSA21 was produced maximum yield of alkaline protease and it was selected as a potent strain for further studies. The optimum temperature and pH were determined as 50^{O}C and 9 and best carbon and nitrogen sources were L(-) arabinose and potassium nitrate. This information has enabled the ideal formulation of media composition for maximum protease production by this organism. After optimization, the mass production was carried out in one liter of optimized media at 50 ºC for 48 h at pH of 9 on basal medium. In conclusion, results of the present study suggested the possibility of *Shewanella putrefaciens*-EGKSA21 to produce enzymes by BM using cheapest substrates for enzyme production. This enzyme was stable over a wide range of pH and temperature. Considering the overall properties of different alkaline enzymes of microbial origin and the thermostable alkaline enzyme from our strain

Shewanella putrefaciens-EGKSA21 is better as regards to pH and temperature**.**

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