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Arab Univ.
J. Agric. Sci.,
Ain Shams Univ.,
Cairo, 19(1), 179-189, 2011

MICROBIAL PRODUCTION OF THERMOALKALIPHILIC ENZYMES FROM EL-KHORMA GOVERNORATE FOR APPLICATION IN BIODETERGENT TECHNOLOGY

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Keywords: Thermostable, Alkaliphilic, Protease, Biodetergent, *Bacillus licheniformis*

ABSTRACT

Detergents are an undetectable source of pollution, which hidden in most of our daily activities. Detergents could cause harmful effects before they are completely degraded. It is wise to reduce the amount of detergents that usually used by invention new bio-friendly formula contains efficient enzymes such as protease. Screening studies were carried out for one hundred and fifty bacterial isolates with respect to their ability to produce protease(s), after growing on slaughter house wastes (SHW) isolated from El-Khorma governorate,Taif, Kingdom of Saudi Arabia (KSA) at 55ºC, and pH 9. The most potent thermophilic bacterial isolate concerning of alkaline thermostable protease(s) production was identified as *Bacillus licheniformis* EGT50. Alkaline thermostable proteases productivity by the most potent bacterial isolate was affected by substrate concentrations (solid substrate), carbon source, nitrogen source, amino acid supplements, incubation temperature, incubation period, and inoculum size. Maximum both enzymes production by *B. licheniformis* EGT50 was obtained on SHW concentrations, 7.5 %; galactose; diammonium hydrogen phosphate; arginine at 55°C for 72 h. when inoculated by 0.5 ml. The protease production under all optimal conditions was increased many folds from 563.68 to 17825 U/ml (31 fold). The purification fold of *B. licheniformis* EGT50 alkaline thermostable protease increased to 394.7 after applying Sephadex G200 column chromatography techniques. The enzyme productivity of protease has been determined and the result proved the possibility to use the crude and purified enzymes in biodetergent technology.

1. INTRODUCTION

Enzymes play a significant role in our life. Their existence had been associated with the history of ancient civilizations. Enzymes from plant and microorganisms had been used in brewing, baking, alcohol production, cheese, vinegar making….. etc. The uses of enzymes were variable ranging from just making wine or bread to producing complicated fermentations processes. Technical enzymes represent 1 billion USD in 1999 **(Schäfer *et al* 2005; Kumar *et al* 2009 and Haddar *et al* 2010).** Part of these enzymes is the thermostable enzymes, which are better, suited for harsh industrial processes and constitute more than 65% of the global market **(Leuschner and Antranikan, 1995; Rao *et al* 1998; Gupta *et al* 2002; Beg and Gupta, 2003; Amara *et al* 2009; Mário *et al* 2009; Akanbi *et al* 2010; Haddar *et al* 2010 and Eltayib *et al* 2010).**

Protease constitutes one of the most important groups of industrial enzymes, accounting for about 60% of the total enzyme market **(Nunes and Martins, 2001)**. Protease are of commercial value and find multiple applications in various industrial sectors. Proteases are widely used in detergents, food

(Received December 1, 2010)
(Accepted December 26, 2010)

and leather tanning industries **(Abidi *et al* 2008)**. Alkaliphilic *Bacillus* are considerable as prolific producers of alkaline proteases, which exhibit significant activity and stability at high pH and temperatures **(Yang *et al* 2000; Christiansen and Nielsen, 2002; Joo *et al* 2003 & 2004 and Kumar *et al* 2009**). Enzymes have many applications especially in paper industry, detergents, drugs, degradation of different wastes, textile, food, pharmaceutical, leather, degumming of silk goods, manufacture of liquid glue, cosmetics, meat tenderization, cheese production, growth promoters …etc **(Schäfer *et al* 2005; Cowan,1996 and Haddar *et al* 2010**). Meanwhile one of the most important and profitable applications for enzymes is in detergents. The first use of enzymes in detergents occurred in 1913 when Röhm and Haas introduced crude trypsin into their detergent. Burnus based on a German patent issued to **Otto Röhm (1913) (Schafer *et al* 2005)**. To provide desirable benefits, enzymes must be stable and function well in the presence of a variety of potentially unfriendly detergent ingredients (e.g., anionic/ nonionic/ cationic surfactants, chelants, builders, polymers, bleaches) and in various forms of detergent products (i.e., liquids and powders) **(Schäfer *et al* 2005; Amara *et al* 2009; Mário *et al* 2009 and Akanbi *et al* 2010)**.

In present study, isolation, purification, and identification of thermophilic bacterial isolates from El-khorma governorate, Kingdom of Saudi Arabia; production of an alkaline thermostable protease from *B. licheniformis* EGT50 and optimization of the alkaline thermostable protease production parameters for potential use as a detergent industry.

2. MATERIALS AND METHODS

1- Microorganisms: An alkaliphilic *B. licheniformis* EGT50 strain, which produces proteases, was originally isolated from different desert soil samples collected from different localities of El-khorma governorate, Taif, kingdom of Saudi Arabia. The most potent thermoalkaliphilic strain found to be capable of producing extracellular alkaline protease was identified and the criteria laid down in Bergey's Manual of Systematic Bacteriology **(Sneath, 1986; and Schallmey *et al* 2004)**.

2- Construction of standard enzyme and protein curves: A stock solution of (50'000 µg/ml) purified protease enzyme supplied by Sigma chemicals Co. was prepared in Tris-HCl buffer (0.2M) at pH 9, where descending dilutions were prepared. After preparing the required dilutions for protease, only 0.1 ml of each dilution was transferred to each well in the gelatin–substrate medium using gelatin clearing zone (GCZ) technique. The obtained standard curve was used for estimating the enzymes activities in terms of µg/ml and then translated into 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 time under all the specified conditions of enzyme assay (clearing zone technique). The total protein determination was made according to the method of **Lowry *et al* (1951)** using bovine serum albumin as a standard protein

3- Production medium: Alkaline thermostable protease production was determined by applying a modified basal medium given by **Vincent (1970)**, containing of the following ingredients (g/l): SHW, 10; NaCl, 6; $(NH_4)_2SO_4$, 1; yeast extract, 1; KH_2PO_4, 0.5; $MgSO_47H_2O$, 0.1; $CaCl_26H_2O$, 0.1; $FeSO_47H_2O$. All ingredients were dissolved in distilled water and completed up to one liter. The initial pH of the culture medium was adjusted at 9.

4- Protease(s) assay medium for gelatin clearing zone (GCZ) technique: This medium was devoted to gelatin clearing zone (GCZ) technique according to **Ammar *et al* (1991).** The assay plates contained 1% gelatin and 1.5% agar for solidification, to be dissolved in 100 ml of Tris-HCl buffer (pH 9). At the end of incubation period, protease(s) activity was detected by flooding each plate with 10 ml freshly prepared acid mercuric chloride solution **(Barrow and Feltham, 1993)**. Mean diameters of clearing zones were measured, and taken as indication for proteolytic activities. The standard errors of mean values were less than 3 %.

5-Solid state fermentation (SSF): Five grams of SHW were introduced moistened with 5 ml of production medium in 250 ml Erlenmeyer flask, and autoclaved at 15 psi for 20 min, was taken as the basal medium for SSF studies was adjusted at pH of 9.0. Medium was left to cool, inoculated with 0.5 ml of the inoculum (A 600; 0.8), having 1.5 x 10^6 cells /ml in case of *B. licheniformis* EGT50 from 12 h. old shake culture and incubated at 65°C. Visual observations regarding growth were made each day and the SHW was mixed with 10 ml of tap water, and filtered through a metallic sieve. The extracted filtrate was centrifuged (10'000 xg; 4°C) for 15 min and cell free filtrate was used as the source of crude alkaline thermostable protease.

6- Optimization of fermentation conditions for alkaline thermostable protease production: Protease production was optimized under SSF on SHW**,** unless otherwise stated, by altering various physicochemical conditions. Effect of different temperatures, pH values, different substrate concentrations, various supplements on the alkaline thermostable protease production by adding different carbon sources (1% w/w), nitrogen sources, inoculum sizes and incubation periods were carried out by allowing the *B. licheniformis* EGT 50 strain to grow on SHW and incubated at different incubation periods at 55ºC at pH 9.

7- Protease production by *B. licheniformis* EGT50: *B. licheniformis* EGT50 was allowed to grow under the optimal static natural substrate under solid state fermentation conditions on slaughter house wastes for protease(s) production. The optimum protease(s) production medium contained (g/l, w/v), 10g of slaughter houses wastes per flask of 1000 ml capacity were used and supplemented by 20 ml of production medium, which contained (g/l, w/v): NaCl, 6; KH_2PO_4, 0.5; $MgSO_4 7H_2O$, 0.1; $CaCl_2 6H_2O$, 0.1; yeast extract, 1 in addition to galactose, ammonium dihydrogen phosphate, and arginine, pH was adjusted at 9, and inoculated with bacterial suspension and incubated at 55ºC for 72 h.

8- Enzyme purification: To the cell free supernatant, ammonium sulphate was added up to 80 % saturation and centrifuged (15'000 rpm for 15 min) after 2 h of incubation at 4°C. The precipitate was dissolved in a minimum amount of Tris-HCl buffer (0.2M) at pH 9, dialyzed overnight against the same buffer and retained for further purification steps by sephadex G200. After dialysis, the supernatant, containing enzyme protein, was applied to a sephadex G200 (particle size 200M) column (50 cm x 2.5 cm) pre-equilibrated with Tris-HCl buffer (0.2M) at pH 9. Fractions (5 ml each) were collected at the flow rate of 20 ml /h and assessed for enzyme activity.

3. RESULTS AND DISCUSSION

Enzymes have long been of interest to the detergent industry for their ability to aid in the removal of proteinaceous stains and to deliver unique benefits that cannot otherwise be obtained with conventional detergent technologies. Due to potential usefulness of alkaline thermostable protease in bio-detergent industry, the development of methods for cheaper production of enzyme is very important. SSF holds tremendous potential for the production of enzymes and it can be used of special interest in these processes where the crude fermented product may be used directly as enzyme source **(Tongerdy, 1998; Pandey *et al* 1999 a&b; Haddar *et al* 2010 and Eltayib *et al* 2010)**.

Out of one hundred and fifty thermophilic bacterial isolates was found that only 20 isolates gave higher protease productivity. Data recorded in **Table (1)** showed the ability of twenty bacterial isolates selected from the qualitative screening to attack SHW for protease(s) production. It was found that bacterial isolates number EGT50, EGT95, EGT106 and EGT147 gave the highest proteolytic productivities 28; 22.16; 20.5 and 20.8 mm respectively. From the previous results, bacterial isolate viz. EGT50 was selected as the most potent bacterial isolates for their potentiality to highest production of alkaline thermostable protease.

3.1. Optimization of solid state fermentation (SSF)

There are several factors, affecting SSF processes for protease production. Among these, selection of a suitable strain, and selection of process parameters (Temperature, pH values, substrate concentration, nitrogen sources and incubation periods) are crucial **(Pandey *et al* 2000)**. While efforts largely continued to exploit filamentous fungi and yeast for the production of various products, attempts have also been made to explore the possibilities of using bacterial strains in SSF systems **(Babu and Satyanarayana, 1995)**. The most potent producer bacterial strain viz. *B. licheniformis* EGT50 for concerning alkaline thermostable protease, used in present manuscript was capable of growing at 55°C and pH 9.

From industrial point of view, in order to get production of low cost of enzymes, this bacterial isolate under study was allowed to grown on natural substances such as SHW under SSF conditions, However, the selection of the previously mentioned substrate for the process of enzymes biosynthesis was based on the following factors viz (i) they represent the most cheapest agro-industrial wastes in Taif; (ii) they are available at any time of the year; (iii) Their storage represents no problem in comparison with other substrates and (iv) they resist any drastic effect due to the exposure to other environmental conditions e.g. temperature, variation in the weather from season to season and or from day to night.

Table 1. Screening program of protease production by the most potent thermophilic bacterial isolates by growing on SHW at 55°C for 48 h. using GCZ technique

No.	Code number	Proteases(s) production GCZ technique (mm).	No.	Code number	Protease(s) production GCZ technique (mm).
1	EGT15	20.0 ±0.13	11	EGT 78	21.8±0.0
2	EGT 38	18.5 ± 0.0	12	EGT 80	18.0±0.47
3	EGT 42	19.5 ±0.0	13	EGT 89	20.6±0.0
4	EGT 47	22.6 ±0.0	14	EGT 90	18.16±0.0
5	EGT 50	28.0±0.0	15	EGT 95	22.16±0.52
6	EGT 60	20.0±0.1	16	EGT 106	20.5±0.49
7	EGT 64	20.0±0.0	17	EGT 108	17.5±0.0
8	EGT 70	19.16±0.0	18	EGT 110	19.0±0.0
9	EGT 71	20.0±0.0	19	EGT 141	20.3±0.58
10	EGT 72	18.6±0.0	20	EGT 147	20.8±0.0

Interestingly slaughter house wastes are an important pollutant factor for the environment, many pathogenic microorganisms can grow on it, this may cause many diseases for man and animals, thus its use for enzymes production help in prevention disease distribution**.** Therefore, the purpose of the present work is to determine the best factors controlling the enzyme(s) productivities by *B. licheniformis* EGT50**.** On the other hand, the economic feasibility of the microbial enzymes production for its application generally depends on the cost of its production processes. In order to obtain high and commercially viable yields of alkaline-thermostable enzymes, it was essential to optimize the fermentation medium used for bacterial growth and enzymes production from the most potent thermophilic *Bacillus* strain. Optimal parameters of the alkaline-thermostable enzymes biosynthesis from microbial origin, varied greatly, with the variation of the producing strain, environmental, and nutritional conditions**.**

The maximum protease(s) productivity was attained at 55°C in the presence of SHW viz. 563.68 U/ml for *B. licheniformis* EGT50 bacterial strain **(Fig. 1)**.

pH is among the other most important factors for any fermentation process and dependent on the type of the moistening agent used in the medium. Each microorganism possesses a pH range for its growth and activity with an optimal value in between the range. The pH of the culture medium plays a critical role for the optimal physiological performance of the microbial cells and the transport of various nutrient components a cross the cell membrane aiming at maximizing the alkaline enzymes yields. Thus, the pH of the fermentation medium has a marked effect on the cell growth and enzyme production **(Kumar *et al* 1999&2004; Kumar *et al* 2009 and Mário *et al* 2009).** Furthermore the optimal pH values may be affected by the incubation temperature in many thermophiles. However, it was not surprising to find that, not only the incubation temperature was affecting the optimal pH value, but also many factors in the environment may change it i.e. secretion of alkaline solutions like ammonia or acids like oxalic acid in the medium **(St. Leger *et al* 1999)**, incubation period, growth changes in medium, growth factors supply, the different minerals and nitrogen source. The variation in the pH value for protease production was affected by strain or species difference. Moreover, several workers indicated that, the variations in the pH value for proteases production even within the same bacterial species owing to the difference in method applied and different environmental conditions. The optimum initial pH value capable of promoting protease(s) production by *B. licheniformis* EGT50 was found to be at the value of 9.0 since the enzyme(s) yields reached up to 447.74 U/ml **(Table 2)**. The maximum protease(s)

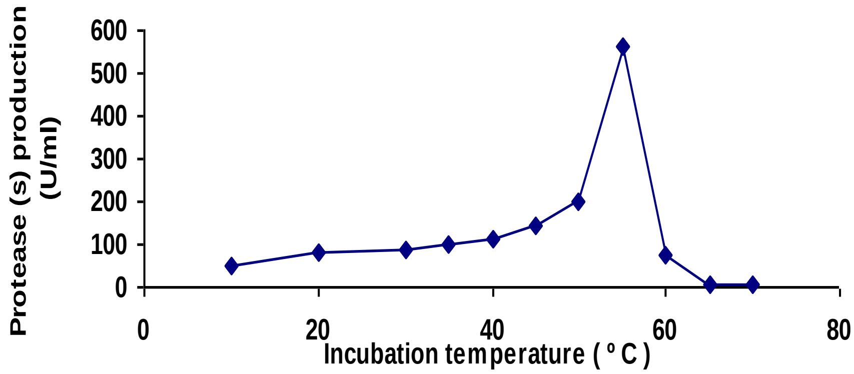

Fig. 1. Effect of different incubation temperatures on protease(s) productivity by *B. licheniformis* EGT50.

Table 2. Relation of different incubation temperatures, pH and substrate concentration to protease(s) production by *B. licheniformis* B-50 allowed to grow on SHBM under Submerged fermentation (SmF) conditions

Incubation temperature (°C)	Protease(s) production (unit/ml)	Initial pH value	Protease(s) production (unit/ml)	Substrate concentration (g/flask)	Protease(s) production (unit/ml)
10	50.23± 2.08	3	0.0	0.05	2.82± 0.9
20	79.62± 1.5	5	10.02 ± 0.0	0.1	13.36 ± 0.52
30	89.33± .38	6	17.82 ± 0.0	0.2	44.77 ± 0.49
35	100.2 ± 0.0	7	100.23± 0.0	0.5	89.33 ± 0.7
40	112.47± 3.09	7.5	178.25± 0.0	1.0	188.8 ± 0.38
45	141.59 ± 0.98	8	316.98± 0.0	1.5	316.98 ± 0.6
50	200.0 ± 2.3	8.5	422.69± 0.0		
55	563.68 ± 2.0	9	447.74±1.04		
60	75.16± 1.4	9.5	10.02± 0.0		
65	4.23± .52	10	5.64 ± 0.0		
70	3.16 ± 0.0				

productivity was reached up to 316.98 u/ml with SHW concentration of 1.5 g/100ml produced by *B. licheniformis* EGT50 by incubation at 55°C for 48 h. These results mean that, protease(s) biosynthesis depends not only on the substrate concentration but also on the producing strain. *B. licheniformis* B42 was able to utilize D (+) galactose which increased protease(s) production on SHW basal medium **(Table 3)**.

Ammonium di-hydrogen phosphate was considered to be the best induced for the highest protease productivity by *B. licheniformis* EGT50 where the enzymes productivity reached up to 2377 U/ml with SHW **(Table 4)**. The maximum protease(s) productivity was reached up to 1888.12 U/ml at inoculum size of 0.5 ml/flask by *B. licheniformis* EGT50. The most potent bacterial strain viz. *B. licheniformis* B50 exhibited its maximum ability to biosynthesis protease(s) with 72h incubation period, since the productivity reached up to 3556.5 U/ml **(Table 5)**.

The maximum protease(s) production reached up to 25178.5 U/ml by introducing thiamin into the production medium, this followed by L-Ascorbic acid and nicotinic acid which reached up to 23770.0 U/ml by *B. licheniformis* EGT50.

The alkaline thermostable protease(s) productivity reached its maximal value 2377.0 U/ml by the addition of L-arginine to SHW production medium by *B. licheniformis* EGT50. Also, DL-serine, and L-glutamine exerted high stimulatory effect on protease production by the same bacterial strain **(Table 6)**.

Table 3. Relation of application of different carbon sources to protease(s) productivity by *B. licheniformis B*-50 allowed to grow under SmF conditions at 55ºC

Carbon source	Protease(s) production (U/ml)	Carbon source	Protease(s) production (U/ml)
Control	316.98± 0.6	Disaccharides	563.68 ± 0.0
Monosaccharides		Maltose	1002.37 ± 0.0
Ribose	1002.37± 0.0	Sucrose	563.68± 0.0
D(+) Xylose	316.98 ± 0.6	Cellobiose	1782.5± 0.0
D(-) Arabinose	316.98± 0.0	Trisaccharides	
D(-) Glucose	1782.5 ± 0.0	Raffinose	56.36 ± 0.16
D(+) Galactose	1957.6 ± 0.38	Polysaccharides	
D(+) Mannose	563.68± 0.3	Starch	178.25 ± 0.0
D(-) Fructose	1002.37± 0.0	Cellulose	1002.37± 0.0
Rhamnose	1002.37± 0.0	Dextrin	1869.7 ± 0.11
Trehalose	1002.37± 0.0	Inuline	1782.5 ± 0.0
Disaccharides		Polyhydric alcohol	
Lactose	316.98 ± 0.28	Mannitol	563.68 ± 0.0

Table 4. Relation of application of different nitrogen sources to protease(s) productivity by *B. licheniformis* EGT50 allowed to grow on SHW under SSF conditions at 55ºC

Nitrogen source	Protease(s) production (U/ml).
Urea	1782.0 ±1.04
Sodium nitrate	422.69 ± 0.52
Potassium nitrate	422.69 ± 0.52
Peptone	1002.37± 0.0
Magnesium nitrate	1002.37± 0.0
Control	1957.6 ± 0.0
Ammonium sulphate	1336.69± 0.52
Ammonium nitrate	1336.89± 0.52
Ammonium molybdate	316.98 ± 1.04
Ammonium dihydrogen phosphate	2377 ± 0.52
Ammonium chloride	1336.69 ± 0.52
Ammonium acetate	1002.37± 0.0
Ammonium monohydrogen phosphate	1782.5± 0.0

Table 5. Relation of different inocula sizes, different incubation periods and vitamins to protease(s) productivity by *B. licheniformis* B-42 allowed to grow under SmF conditions at 55ºC for 48h.

Inoculum size (ml).	Protease(s) Production (U/ml)	Incubation Period (hours)	Protease(s) Production (U/ml)	Vitamin	Protease(s) Production (U/ml)
0.1	100.23 ± 1.04	6	751.67± 0.79	Control	17825.01 ±0.0
0.2	447.74 ± 1.04	12	1336.69± 0.52	Ascorbic acid	23770.0 ±0.79
0.4	597.07 ± 0.9	24	2377 ± 0.52	Nicotinic acid	23770.0±1.3
0.5	1888.12 ± 1.04	48	2825.07 ± 0.38	Thiamine (B_1)	25178.5±0.93
1	1002.37 ± 1.04	72	3556.5 ± 0.42	Pyridoxin	10023.7±0.98
1.5	1002.37 ± 0.0	96	2825.07 ± 0.75	Riboflavin	13366.87±0.52
2	316.98 ± 1.04	120	1336.69 ± 0.71	Folic acid	7096.26±0.0
2.5	178.25 ± 0.0	144	316.98± 1.04		
5	1336.69 ± 0.52				
10	56.36 ± 0.0				

Table 6. Relation of different amino acids application to protease(s) productivity by *B. licheniformis* B-50 allowed to grow on SHW under semi solid fermentation conditions at 55ºC.

Side chain (SC).	Amino acid.	Protease(s) production (U/ml).
Control		1336.7± 0.52
Aliphatic SC	Glycine	316.98± 0.24
	DL-Alanine	316.98± 0.0
	DL-Valin	1336.69± 0.52
	L-Leucine	237.7± 0.52
	DL-Isoleucine	422.69± 0.52
Hydroxylic (OH) Group-containing SC	DL-Serine	2118.5± 0.49
	DL-Threonine	751.67± 0.52
	DL-Tyrosine	751.67± 0.52
Sulphur atom-containing SC	L-Cystein	316.98± 0.0
	L- Methionine	1002.37± 1.04
Acidic groupes or their amides	DL-Aspartic acid	1002.37± 0.52
	L-Glutamine	1888.12± 1.2
Basic group	L-Arginine	2377.0± 0.52
	L-Lysine	563.68± 0.0
	L- Histidine	1002.37± 1.04
Aromatic group	L -Phenylalanine	893.36± 0.38
	Tryptophan	1336.69± 0.52
Imino group	L- Proline	316.98± 0.0

Table 7. A summary of the optimal nutritional and environmental parameters controlling of protease productivities by *B. licheniformis* EGT50 under solid state fermentation conditions

Parameter	*B. licheniformis* B42	Parameter	*B. licheniformis* B42
Temperature (°C)	55	Inoculum size (ml)	0.5
pH value	9	Incubation period (hour)	72
Substrate concentration	1.5	Amino acid	Argenine
Carbon source	Galactose	Bottle capacity (ml)	1000
Nitrogen source	$(NH_4)_2H2PO_4$		

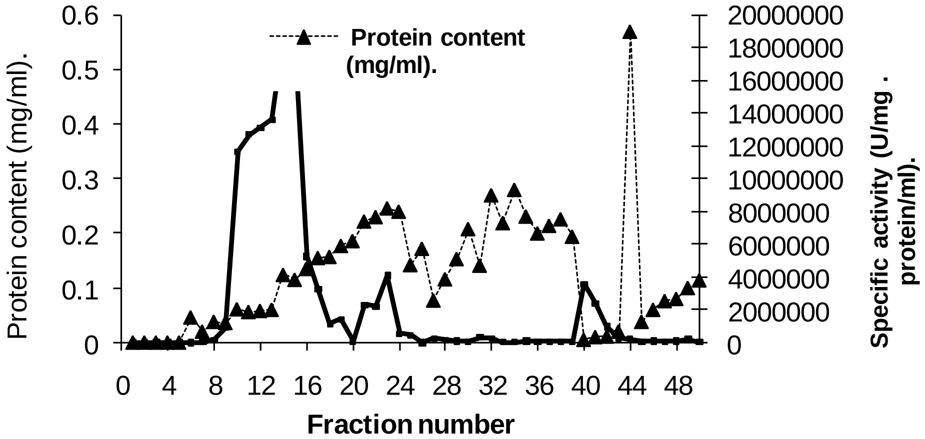

Fig. 2. Fractionation pattern of protease(s) produced by *B. licheniformis* B-42 at 55ºC using sephadex G-200 column chromatography technique.

Table 8. A summary of the purification steps of protease produced by *B. licheniformis* EGT50 allowed to grow on SHW substrate at 55ºC under SmF

Purification step	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg/ml)	Protease activity (U/ml)	Total activity	Specific activity (U/mg, protein)	Purification fold	Yield (%)
CFF	1140	0.38	433.2	17825.01	20320510.94	46907.92	1	100
Ammonium sulphate	100	0.965	96.5	2000000	200000000	2072538.86	44	980
Dialysis against sucrose	4.5	1.1	4.95	5636765.86	25365446.37	5124332.6	109.24	124
Sephdex G-200	5.0	0.124	0.62	2296307.24	11481536.2	18518606.7	394.7	56

Data recorded in **Table (7)** showed a summary of the optimal nutritional and physicho-chemical conditions for alkaline thermostable protease production by *B. licheniformis* EGT50 grown on SHW as preferable substrates.

3.2. Partial purification of the alkaline thermostable enzyme and its kinetic characterization

The application in the bio-detergent industry does not require highly-pure alkaline thermostable protease and generally makes use of crude or partially purified preparations are valid. However, it is significant to obtain enzymes with higher specific activity for their kinetic characterization. Traditionally, the purification of alkaline thermostable proteases from fermentation media has been done in several steps which include centrifugation of culture filtrate, selective precipitation of the enzyme by ammonium sulphate, followed by gel filtration **(Pandey *et al* 2001; Mário *et al* 2009 and Haddar *et al* 2010**).

An attempt to purify alkaline thermostable protease from *B. licheniformis* EGT50 partially, the cell free filtrate supernatant was subjected to ammonium sulphate precipitation, gel filtration on Sephadex G200 columns chromatography. *B. licheniformis* EGT50 was allowed to grow on the production medium under all optimal semisolid fermentation conditions as shown in **Table (7)** for the production of alkaline thermostable protease. At the end of incubation period, 1000 ml of protease(s) was extracted. Results presented in **Figure (2)** showed that three active peaks were obtained after purification of protease(s) by applying sephadex G200 column chromatography in fractions (9-18), fractions (21-26), fractions (39-42) and the fraction 14 reached the highest specific activity up to 18518606.7 U/mg protein.

A summary of the purification steps of protease produced by *B. licheniformis* EGT50 was presented in **Table (8)**. The alkaline thermostable enzyme was partially purified 394.7 folds with sephadex G200.

Conclusion

Bacillus licheniformis was isolated from soil samples collected from different localities of El-Khorma Governorate,Taif, kingdom of Saudi Arabia (KSA). and the organism was studied for the production of thermoalkaliphilic protease under different environmental and nutritional requirements on cheap raw material viz. slaughter house wastes (SHW). This enzyme was found to have ability to stability at high temperature and alkaline pH is useful for many industrial processes especially in biodetergent industry.

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