EXTRACELLULAR ALKALINE PROTEASE PRODUCTION BY *Bacillus licheniformis* IN SUBMERGED FERMENTATION

Ogbonnaya Nwokoro*[1], Ogechi H. Ekwem[2], Uchenna Oyeagu[2]
[I]Industrial Microbiology and Biotechnology Laboratory,
Department of Microbiology, University of Nigeria, Nsukka, Nigeria
[2]South East Zonal Biotechnology Centre, University of Nigeria, Nsukka, Nigeria
*E-mail: ogbonnaya.nwokoro@unn.edu.ng

Abstract
A protease producing Bacillus licheniformis was isolated from abattoir soil sample. Selection of the isolate was based on the production of the highest zone diameter of 39 mm on skim milk agar. In a preliminary experiment, bacterial growth was measured as increase in optical density (OD) of medium at 600nm. There were increases in OD from 0.01 at 0 h to 0.66 after 48 h. Protease production also increased from the time of incubation at which 0.11 Units /mg protein were produced to 51.4 Units /mg protein after 48 h and decreased after 72 h. The best carbon and nitrogen sources for enzyme production were glucose and peptone respectively, while the most effective inorganic nitrogen source was observed to be ammonium chloride. Among the agricultural raw carbon sources used, cane sugar, corn flour, rice flour caused the production of 51.0, 43.2, 46.1 Units/ mg protein respectively after 48 h incubation. Agricultural raw nitrogen sources namely, soy bean meal and corn steep liquor caused the production of best enzyme activities of 50.8 and 42.4 Units/ mg protein respectively. Best enzyme activities were observed in media containing metal ions like Ca^{2+}, Mg^{2+} and Mn^{2+}. The organism produced protease in broad temperature range of 15-60 ºC and pH range of 4.0 to 11.0. The optimum conditions observed for protease production were 35 ºC and pH 9.0. The above results indicate that this isolated bacterium can be useful as a biotechnological tool for industrial purpose.

Keywords: protease; carbon sources; nitrogen sources; *bacillus licheniformis*; submerged fermentation.

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1. INTRODUCTION

Proteases (EC 3.4.21-24 and 99; peptidyl-peptide hydrolases) are enzymes that hydrolyze proteins through the addition of water across peptide bonds and catalyze peptide synthesis in organic solvents and in solvents with low water content (Raj et al., 2012). In all living organisms, proteolytic enzymes are widely found and are essential for cell growth and differentiation (Vadlamani et al., 2011). Proteases constitute the largest group of enzymes in bio-industry with a long array of uses. They play an invincible role in industrial biotechnology, especially in detergent, food and pharmaceutical industries (Jisha et al., 2013). From plants papain and ficin are important proteases, trypsin and chemotrypsin from animals and alkaline proteases from microorganisms like *Bacillus* sp.; *Pseudomonas*; *Penicillium* sp., *Aspergillus* spp. etc. (Ellaiah et al., 2002). Among these, *Bacillus* and *Pseudomonas* spp. are specific to produce extracellular and alkaline proteases while *Bacillus* was found to be predominant and a prolific source of alkaline protease (Deng et al., 2010).

Proteases have got wide range of commercial usage in detergents, leather, food and pharmaceutical industries (Jellouli et al., 2009). They are industrially important enzymes and constitute a quarter of the total global enzyme production (Kalaiarasi et al 2009).

Alkaline proteases are of considerable interest in view of their activity and stability at alkaline pH. Of all the alkalophilic microorganisms, members of the genus *Bacillus* were found to be predominant and a prolific source of alkaline proteases. Alkaline proteases are a physiologically and commercially important group of enzymes used primarily as detergent additives. *B. firmus* MTCC7728 produces extracellular alkaline protease, with great potential in various industries, and several processes like silver recovery, bioremediation and protein hydrolysate production (Rao and

Narasu 2007). As there is increase in demand of industrial protease, so it is desirable to get new sources of protease with maximum production. Wastes particularly from dairy, meat and poultry processing industries act as sources for large amount of protein-rich materials and were biologically transformed into recoverable products (Raj et al 2012). Owing to their high protein content, they may serve as excellent sources for isolating proteolytic microorganisms (Vishwanatha et al., 2010). This research work focuses on the isolation of proteolytic bacteria from abattoir soil and the determination of the factors that affect maximum protease production from the selected isolate.

2. MATERIALS AND METHODS

Soil sample was collected at an abattoir into sterile polyethylene bag. A 10 g sample was dissolved in 90 mL of distilled water (pH= 6.5) contained in 500 ml Erlenmeyer flask and thoroughly shaken. Then, 1 mL of the sample was serially diluted in 9 mL 0.1% peptone water diluent. The diluted sample was plated onto Nutrient agar (Oxoid Ltd., UK) and the plates incubated at 35 ^{o}C for 24 h. Pure bacterial cultures were obtained by streaking on fresh agar plates and were assigned arbitrary numbers. A loopfull of each culture was added into 100 mL medium designated as 'E' containing in w/v; sucrose, 1.5 %; casein, 1%; KH_2P0_4, 0.05%; K_2HP0_4, 0.01% and $FeSO_4.7H_2O$, 0.01%. The medium pH was adjusted to 8.2 using sterile sodium hydroxide. The isolates were incubated with shaking in a Gallenkamp orbital incubator for 24 h at 35°C.

Screening and selection of protease producing bacteria

The individual colony was screened for protease production according to the method described by Dam et al., (2013) using 1% skim milk agar plate. The plates were incubated for 24 h at 37^{o}C and then flooded with 10% tannic acid solution and incubated for 24 h at room temperature. Based on the largest clear zone of hydrolysis, the bacterial strain was selected for further use. The selected strain was identified as *Bacillus licheniformis* based on morphological, physiological and biochemical characteristics as described in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

Medium E was supplemented with 1.5% (w/v) each of the following carbon sources: fructose, lactose, glycerol, maltose, glucose, soluble starch, cellulose powder, mannitol, galactose, cane sugar, corn flour and rice flour devoid of sucrose. When sucrose was under test, it was retained in that series of fermentation flasks. The following nitrogen sources at 1% w/v concentration were each added into medium E in place of casein: yeast extract: ammonium chloride, urea, sodium nitrate, gelatin, tryptone, peptone, glutamate, alanine, leucine, beef extract, soybean meal and corn steep liquor. The flasks were autoclaved at 121 ^{o}C for 15 min. and inoculated with 1 x 10^8 cells/mL of *Bacillus licheniformis*. Each flask was incubated in a Gallenkamp shaker at 30^{o}C for 72 h. Samples were withdrawn for analysis at 24 h intervals. The content of each flask was centrifuged at 2515 x g for 15 min. and the supernatant designated as crude enzyme.

Enzyme assay

The protease activity was estimated by the method described by Beg et al., (2003). Following incubation, the bacterial broth was centrifuged at 2515 x g for 20 min. to obtain the cell free supernatant (CFS). Aliquot of 1 mL of CFS was added to 1 mL of 1% (w/v) casein solution in glycine-NaOH buffer of pH 10.5 and incubated for 10 min. at 60^{o}C. The reaction was stopped by addition of 4 mL of 5% trichloroacetic acid. The reaction mixture was centrifuged at 2515xg for 10 min and to 1 mL of the supernatant 5 mL of 0.4 M Na_2CO_3 was added followed by 0.5 mL Folin-Ciocalteu reagent. The amount of tyrosine released was determined using a spectrophotometer at 660 nm against the enzyme blank. One unit of protease activity was defined as the amount of enzyme required to release 1 µg of tyrosine per mL per min. under the assay conditions.

Partial purification of the enzyme:
The crude enzyme was dialyzed overnight against 0.1M Tris/HCl buffer (pH 8.2). Solid ammonium sulphate was added to the crude enzyme extract to 70% saturation, incubated for 10 h with gentle mixing. The solution was centrifuged at 2515 x g for 15 min. and the supernatant was dissolved in 0.1M Tris/HCl buffer (pH 8.2) and dialyzed overnight against the same buffer. The dialysate was used as enzyme solution.

Effect of metal ions on enzyme production:
The metal ions (0.01 % w/v) were each added into medium E in place of $FeSO_4.7H_2O$. The media were incubated for 24 h in a Gallenkamp orbital incubator followed by centrifugation at 2515 x g for 15 min. The supernatant was assayed for enzyme activity.

The effect of pH on enzyme activity
The effect of pH on activity of the protease was determined by using buffer solutions of different pH (citric acid/sodium citrate buffer, pH 4.0-6.0; potassium phosphate buffer, pH 7.0 to 8.0; Tris/HCl buffer, pH 8.1-9.0 and carbonate/bicarbonate buffer (pH 9.0-11.0) for enzyme assay. The pH activity profile of the enzyme was determined by incubating 1 mL of enzyme solution with 1 mL of 1% (w/v) casein solution prepared in buffers of different pH values (5.0-11.0) and incubated for 2 h at 60^oC. The reaction was stopped by the addition of 4 mL of 5% trichloroacetic acid. The reaction mixture was centrifuged at 2515 x g for 10 min and to 1 mL of the supernatant 5 mL of 0.4 M Na_2CO_3 was added followed by 0.5 mL Folin-Ciocalteu reagent. The amount of tyrosine released was determined.

The effect of temperature on enzyme activity
The temperature activity profile of the enzyme was determined by incubating 1 mL of enzyme solution with 1 mL of 1% (w/v) casein solution prepared in 0.1M Tris/HCl buffer (pH 8.2) and incubated for 2 h at different temperatures (15, 20, 25, 30, 35, 40, 45, 50, 55 and 60^oC) in a thermo static water bath (Kottermann, Bremen, Germany). The reaction was stopped by the addition of 4 mL of 5% trichloroacetic acid. The reaction mixture was centrifuged at 2515 x g for 10 min and to 1 mL of the supernatant 5 mL of 0.4 M Na_2CO_3 was added followed by 0.5 mL Folin-Ciocalteu reagent. The amount of tyrosine released was determined.

Analysis
Determination of pH was done with a glass electrode pH meter (PYE Unicam, England). Protein content was estimated by the method of Lowry *et al.*, [1951] using bovine serum albumin (Sigma-Aldrich) as a standard.

3. RESULTS AND DISCUSSION

A total of thirty-one bacterial isolates from abattoir soil were plated on 1% skim milk agar. The organisms showed various degrees of hydrolysis on the agar (Table 1). The best protease producing strain was selected for further studies depending on the highest clear zone on the agar. It was found that the bacterial strain CE 21 showed largest zone clearance of 39 mm. Based on its morphological, physiological and biochemical characteristics, the selected strain was identified as *Bacillus licheniformis.*
Results in Table 2 shows bacterial growth and protein production in medium E. Growth measured as increase in optical density of medium at 600nm increased from 0.01 at 0 h to 0.66 after 48 h and decreased to 0.40 after 72 h. Enzyme production also increased from the time of incubation at which 0.11 Units /mg protein was produced to 51.4 Units /mg protein after 48 h and decreased after 72 h incubation.
Results of the effects of carbon sources on protease activity shows that the different carbon sources had various effects on production of alkaline extracellular protease by *Bacillus licheniformis*.
Glucose was found the optimum source and caused the production of enzyme activity of 31.2 U/mg protein after 24 h. This level peaked at 61.9 U/mg protein after 48 h and decreased thereafter to 42.7 U/mg protein after 72 h (Table 3).

Table 1: Selection of fungal isolates based on their protease activities

S/N	Bacterial isolate	Clear zone diameter (mm)	Gram reaction
1	CE 1	12	+
2	CE 2	4	+
3	CE 3	15	+
4	CE 4	8	-
5	CE 5	8	-
6	CE 6	18	-
7	CE 7	23	+
8	CE 8	15	-
9	CE 9	3	+
10	CE 10	6	+
11	CE 11	17	+
12	CE 12	19	+
13	CE 13	22	-
14	CE 14	28	-
15	CE 15	6	+
16	CE 16	23	-
17	CE 17	4	+
18	CE 18	16	+
19	CE 19	11	+
20	CE 20	30	-
21	CE 21	39	+
22	CE 22	28	-
23	CE 23	4	-
24	CE 24	15	-
25	CE 25	17	-
26	CE 26	9	-
27	CE 27	13	+
28	CE 28	10	-
29	CE 29	6	+
30	CE 30	29	+
31	CE 31	5	-

Table 2: Bacterial growth and protease production in medium E

Time (h)	OD $_{600\ nm}$	Enzyme activity (U/mg protein)
0	0.01	0.11
24	0.18	32.7
48	0.66	51.4
72	0.40	36.2

Cultural and environmental conditions play important roles in microbial growth and enzyme production. The present study was aimed for optimization of medium components for the maximum production of alkaline protease. Thumar and Singh (2007) reported that glucose was best carbon source for growth but protease production was optimal with sucrose for actinomycetes.

Table 3: Effect of carbon sources on the production of protease by *Bacillus licheniformis*

Carbon source (1.5 %, w/v)	Period of incubation (h)		
	24	48	72
	Protease Activity (Units/mg protein)		
Lactose	16.8	37.3	18.3
Fructose	17.4	37.4	26.6
Glycerol	29.0	46.3	40.1
Sucrose	32.7	51.4	36.2
Maltose	18.7	41.8	35.8
Mannitol	17.2	40.5	36.8
Glucose	34.2	61.9	42.7
Soluble starch	14.5	28.9	21.4
Cellulose powder	8.5	21.6	14.6
Galactose	16.0	29.2	14.9
Cane sugar	28.8	51.0	40.3
Corn flour	26.5	43.2	30.6
Rice flour	19.4	46.1	37.2

This present investigation is in line with the findings of (Boominadhan et al., 2009) who reported maximum protease enzyme activity in glucose medium among other carbon sources and beef extract among other nitrogen sources for *Bacillus* spp. Also, glucose supported the maximum production of protease while the best nitrogen source for protease production was beef extract (Ariole and Ilega 2013).

The best carbon source for enzyme production in *Bacillus pumilus* SG 2 was glucose and the best nitrogen sources were yeast extract and casein (Sangeetha et al. 2008). *Bacillus cereus* strain 146 which used glucose for maximum amount of alkaline protease production (Shafee *et al*., 2005). The study of alkaline protease production showed that glucose and casein were the best carbon and nitrogen sources respectively (Basavaraju et al., 2014). Afify et al [2009] reported that glucose resulted in the reduction in protease production which they attributed to catabolite repression by high glucose available in the medium. Increased yields of alkaline proteases were reported by several other workers who used lactose,

sucrose and fructose (Nejad et al., 2010). Yang *et al.* (2000) reported that lactose or arabinose enhanced protease production by *Bacillus subtilis*.

There is an increasing interest in the use of agricultural raw materials and wastes products because they are cheap carbon and energy sources not only to cultivate microorganisms, but also to obtain valuable products which have different applications with economic significance (El-Enshasy *et al.,* 2008). In this present investigation, cane sugar, corn flour and rice flour were used as carbon sources in medium E for protease production. Highest protease levels of 51, 43.2 and 46.1 Units/ mg protein respectively produced from these substrates after 48 h and these levels were comparable to the ones produced with pure carbon sources (Table 3).

Testing the effect of various nitrogen sources on protease production (Table 4), it was found that peptone gave the highest enzyme activity of 58.6 Units/ mg protein after 48 h. Another notable finding of the study was that the inorganic nitrogen sources namely ammonium chloride, sodium nitrate and urea proved to be inferior compared to the organic nitrogen sources for protease production. We tried combination of glutamate+peptone, alanine+peptone and leucine+peptone as nitrogen sources. The combinations were better than single nitrogen sources (Table 4). Roughly the same amount of extracellular protease was produced as compared to peptone when soy bean meal and corn steep liquor were individually used as nitrogen sources. The production of the enzyme with these substrates would be cheap and economically attractive. Many studies used natural sources such as rice bran, soy bean, wheat flour, wheat bran, corn bran, corn starch and orange peels as medium constituents to support protease production by bacteria (Joo and Chang, 2005). Study by Sehar and Hameed (2011) concluded that organic nitrogen sources such as casein, gelatin, peptone, yeast extract and beef extract had significant effects on extracellular protease production by a halophilic *Bacillus* sp, whereas simple inorganic nitrogen sources in the form of ammonium compounds showed reduced growth and protease production.

Table 4: Effect of nitrogen sources on the production of protease by *Bacillus licheniformis*

Nitrogen source (1 %, w/v)	Period of incubation (h)		
	24	48	72
	Protease activity (Unit/mg protein)		
Ammonium chloride	20.9	36.1	22.8
Urea	14.7	19.8	8.9
$NaNO_3$	10.5	22.9	20.6
Tryptone	25.2	40.1	34.3
Yeast extract	20.9	53.4	26.9
Peptone	28.1	58.6	49.3
DL-glutamate	29.3	55.6	30.9
DL-alanine	20.0	49.8	41.2
DL-leucine	34.8	56.1	41.8
Beef extract	22.5	49.0	36.7
Soybean meal	33.9	50.8	46.4
Corn steep liquor	29.6	42.4	36.0
Gelatin	18.4	41.6	30.1
Casein	32.7	51.4	36.2
DL-glutamate+peptone	43.2	84.8	58.5
DL- alanine+peptone	39.5	71.6	49.6
DL-leucine+peptone	49.8	75.9	50.5

Table 5: Effect of metal ions on the production of protease by *Bacillus licheniformis*

Metal salt (0.01 % w/v)	Specific activity (Units/mg protein)	% Relative activity
Unsupplemented medium E	36.8	100
$CuSO_4$.$7H_2O$	36.1	98
$MgSO_4$.$7H_2O$	79.5	216
$CaCl_2$	97.9	266
EDTA	4.1	11
$AgNO_3$	30.7	83
$CoCl_2$	10.3	28
$BaCl_2$	28.3	77
$FeSO_4$.$7H_2O$	32.7	89
$ZnSO_4$	26.1	71
$MnCl_2$	76.9	209

Beef extract among the different organic nitrogen sources and ammonium carbonate among the different inorganic nitrogen sources led to a high proteolytic activity by *Bacillus* sp. at 48 h incubation (Rajkumar et al., 2010). Raj et al., (2012) tested the effect of various nitrogen sources on protease production, and found that yeast extract gave the highest enzyme activity. Atalo and Gashe (1993) showed that yeast extract and peptone induced the alkaline protease production.

The addition of $CaCl_2$ in the growth medium resulted in the best accumulation of proteolytic activity (Table 5). The metal chelator ethylene diamine tetra acetic acid (EDTA) led to rapid enzyme inactivation. Calcium was found to be required for the stability of several extracellular proteases including neutral protease (Kumar and Takagi 1999) and alkaline protease (Smita et al., 2012). The most commonly used metal ions are Ca^{2+}, Mg^{2+} and Mn^{2+}. Ca^{2+} ion is also known to play a major role in enzyme stabilization by increasing the activity and thermal stability of alkaline proteases at higher temperatures (Kumar 2002). Other metal ions such as Ba^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, Fe^{3+} and Zn^{2+} are also used for stabilizing proteases (Rattray et al., 1995). Hg^{2+}; Cu^{2+}, Ag^{2+}, Fe^{2+} and Zn^{2+} were found inhibitory to majority of proteases (Moallaei, et al., 2006; Pena-Montes et al., 2008).

The influence of initial pH on protease production was tested over a wide range from pH 4.0 to 11.0 (Fig. 1).

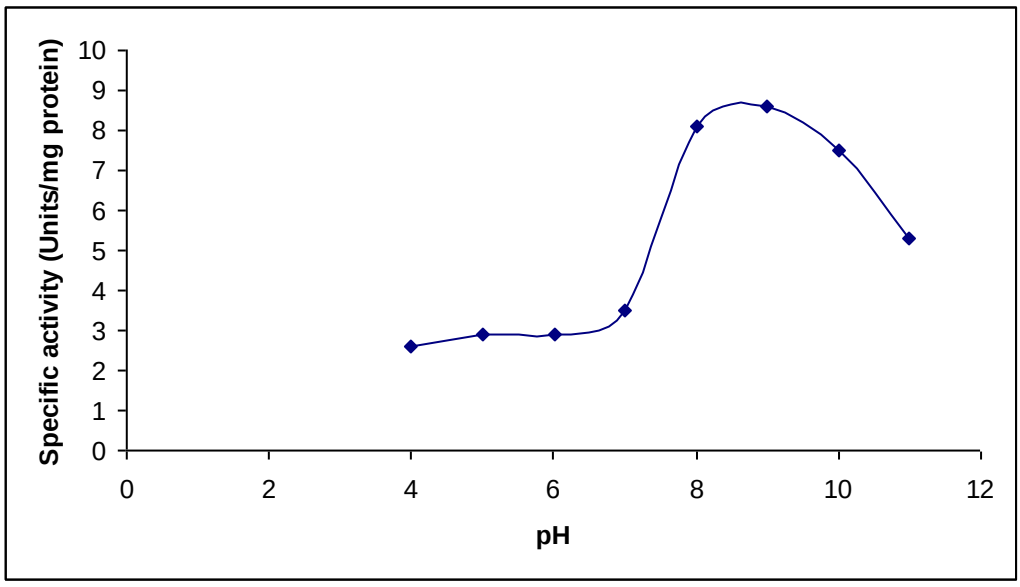

Fig 1: Effect of pH on the production of protease by *Bacillus licheniformis*

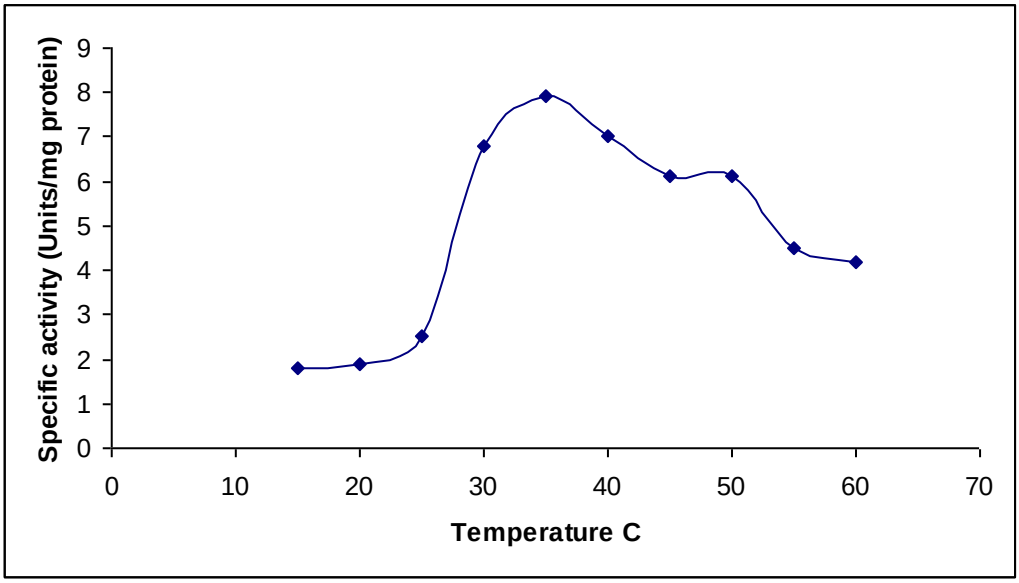

Fig 2: Effect of temperature on the production of protease by *Bacillus licheniformis*

Optimal pH for the enzyme production was 9.0 at which enzyme activity of 8.6 Units/mg protein was produced. This activity level was followed closely by pH 8.0 at which enzyme activity of 8.1 Units/mg protein was produced. Lowest activity of 2.6 Units/mg protein was produced at pH 4.0. The pH of the production medium play a critical role for the optimal physiological performances of the bacterial cell and the transport of various nutrient components across the cell membrane aiming at maximizing the enzyme yields (Smita et al., 2012). Bacterial alkaline proteases were characterized by their high activity at alkaline pH and broad substrate specificity (Mukesh Kumar et al., 2012). The optimum pH for maximum protease production from Bacillus SNR01 was pH 7.0 (Josephine et al., 2012). *Pseudomonas aeruginosa* showed maximum protease activity at pH 9.5, temperature 37ºC and 48 h incubation time (Samanta et al., 2012). Kobayashi *et al.* (1996) reported that optimal activity of bacterial protease at pH 11 while Kumar (2002) reported that the production of an alkaline protease was best at pH 11.5 for *Bacillus* sp. High protease production was recorded after an incubation time of 96 h by *Bacillus subtilis*. Raj et al., (2012) detected protease production over a broad pH range from 4.0 to 9.0, and the maximum enzyme production was noted at neutral pH. Maximum protease activity was observed at pH 9 (Ariole and Ilega 2013). Akujobi, et al., (2012) reported a maximum alkaline protease production at pH 9 for *Pseudomonas aeruginosa* isolated from abattoir soil. The optimum pH for activity of protease produced by *Bacillus* sp. SP5 was 9.0. This observation is comparable with the findings of (Geethanjali *et al.* 2011) using *Bacillus subtilis* where the optimum pH of the medium was 9.0. The alkaline proteases produced from *Bacillus* sp. showed maximum activity at pH 8.0 to 9.0 (Nascimento and Martins, 2004).

The enzyme was incubated over the temperature range from 15 to 60°C (Fig. 2). It was found that temperature of 35 °C was best for enzyme production. At 15 °C, protease was produced while at higher temperature of 55 and 60 °C only were produced. Temperature regulates the synthesis and secretion of extracellular proteases by microorganisms (Balaji 2012). Temperature significantly regulates the synthesis and secretion of bacterial extracellular proteinase by changing the physical properties of the cell membrane (Balaji et al., 2012). Therefore, temperature is an important parameter that should be controlled in order to obtain an optimal enzyme production. The incubation temperature of a fermentation process has a profound role to play on the growth and in turn on the metabolic activities of the microbial cells (Sharma et al.,

1980). The optimum temperature for alkaline protease by *Bacillus* sp. MIG was found to be 30 °C (Mohapatra *et al.* 2003). Many reports showed bacterial and fungal alkaline protease production at lower and moderate temperatures (25-50°C) (Kumar and Takagi, 1999).
Studies report that different species of *Penicillium* including *P. citrinum*, *P. perpurogerum* and *P. funculosum* gave highest yield of protease when incubated at 30°C (Sharma *et al.*, 1980). Haq *et al*. (2004) have also reported that maximum production of protease by *P. griseoroseum* was obtained at an incubation temperature of 30°C and the enzyme production was reduced when the incubation temperature was increased above 30°C. Ariole and Ilega (2013) found temperature of 37°C to be optimum for maximum protease activity. An increase in protease production with increase in temperature up to the temperature of 37°C for *Pseudomonas aeruginosa* from abattoir soil has been reported by (Kalaiarsi and Sunitha, 2009) and [Akujobi et al., 2012] respectively. The optimum temperature for alkaline protease production by *Bacillus* isolates was found to be 37°C (Basavaraju et al., 2014) although it grew and produced alkaline protease in the range of 30 to 40°C). In concurrence to our result are the previous findings, where the bacterial isolates like *Pseudomonas aeruginosa* MTCC 7926, *Serratia liquefaciens* preferred 37 °C for maximum production of protease (Patil et al., 2011). The production of alkaline protease by Bacillus halodurans was investigated, wherein the maximal cell growth was seen at 50 and maximum enzyme production was found at 37ºC (Ibrahim and Al-Salamah 2009).

4. CONCLUSIONS

Bacterial strains from the albattoir effluent were screened for extracellular alkaline protease production. The isolates were inoculated on using 1% skim milk agar plate and produced clear zone around the colony indicating protease activity. Isolate CE 21 produced highest activity and was identified as *Bacillus licheniformis* by biochemical, physiological and cultural characterization. Cultural environment for protease production was optimized using submerged fermentation and it was found that glucose and peptone were the best carbon and nitrogen substrates respectively for enzyme production. Agricultural raw materials when used as carbon or nitrogen also caused the production of significant amounts of protease. Overall best yields were obtained when amino acids were combined with peptone as sources of nitrogen. The pH 9.0, temperature of 35ºC and the addition of $CaCl_2$ enhanced protease production.

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