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Production of Protease by *Bacillus* sp. N-40 Isolated from Soil and Its Enzymatic Properties

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ABSTRACT

Fifty four *Bacillus* strains isolated from different soils were screened for protease production. One strain producing maximum protease was selected. In order to enhance the production of protease, the effects of major medium ingredients, such as carbon, nitrogen sources and metal ions, on the production of the enzyme were investigated. Among the carbon sources used, fructose showed the highest potential for the production. The best organic nitrogen source was skim milk. Inorganic nitrogen sources were not **as effective as organic sources. Addition of combine metal ions minimized the enzyme production. Combinations of Ca^{2+} and Mg^{2+} in medium were the best. Both ions were not effective alone. Increased production (51%) of the enyzme was obtained by manipulating the medium composition. The optimum pH and temperature for the purified enzyme activity were 7.0 and 55°C, respectively. The study of its stability showed that the enzyme is stable in the alkaline pH range 6.0-9.0, and at temperatures between 40 and 70 °C. The enzyme was also thermostable (77% at 55 °C for 3 h). The enzyme activity was stimulated by Mn^{2+} and Ca^{2+}. It showed a single band with a molecular weight of 52 kDA by SDS-PAGE.**

Key words: *Bacillus*, Isolation, Protease, Partial purification, Characterization

INTRODUCTION

Proteases refers to a group of enzymes whose catalytic function is to hydrolyze proteins. They are also called proteolytic enzymes or proteinases. Proteases are classified according to their structure or the properties of the active site. There are several kinds of proteases such as serine-, metallo-, carboxyl-, acidic-, neutral-, and alkaline proteases. Proteases are one of the most important group of industrial enzymes, and commercial proteases account for nearly 60% of the total industrial enzyme market. They are widely used in leather processing, detergent industry, food industries, bioremediation process, pharmaceutical, textile industry, waste processing companies, and in the film industry etc. (Rao et al. 1998). Proteases are obtained from plants and animal organs and microorganisms, with the majority obtained from bacteria and fungi. Currently, a large proportion of commercially available proteases are derived from *Bacillus* strains (Mehrato et al. 1999). *Bacillus* proteases are predominantly extracellular and can be concentrated in the fermentation medium. The most studies focused on screening proteases with a criterion set only to increase activity levels (Kim et al. 1994). Selection of the right organism plays a key role in high yield of desirable enzymes. On the other hand, it is a well-known fact that extracellular protease production in microorganisms is greatly influenced by media components, especially carbon and nitrogen sources (McKeller and Cholette 1984), metal ions (Adinarayana et al. 2003). Isolation and characterization of new promising strains using carbon and nitrogen source is a continuous process (Asokan and Jayanthi 2010).

In the present study, *Bacillus* sp. strains isolated from 31 different Turkish soils were screened for proteolytic activity. A strain which have the highest proteolytic activities were determined and some fermentation conditions such as carbon and nitrogen sources and various metal ions on protease production were investigated. A modified medium was defined for high production of protease. Protease was partially purified and characterized.

MATERIALS AND METHODS

Bacterial isolation and screening for proteolytic activity

Soil samples of 31 different cities of Turkey were collected, serially diluted and plated on nutrient agar plates. The isolated pure colonies were examined for *Bacillus* with standart decription of Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons 1974). A total of 54 *Bacillus* sp. were screnned for protease production by using casein agar (0.5% casein in nutrient agar) and skim milk agar (1% skim milk in nutrient agar). Proteolytic activities of *Bacillus* sp. were detected on the basis of appearance of clear zones around the bacterial colonies. The protease positive colonies were obtained a pure cultures and the most potent four strains were selected for quantitative test of protease.

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Protease production

Two culture media used for protease production and compared. One of them contained (% w/v) : glucose-0.1, peptone-1, yeast extract-0.02, $MgSO_4$-0.01, $CaCl_2$-0.01, K_2HPO_4-0.05 (pH 7.0) (Qadar et al. 2009). Other composed of (% w/v) : glucose-1, peptone-0.5, yeast extract-0.3, $MgCI_2$-0.02, $CaCI_2$-0.04 (pH 7.0) (Sangeetha et al. 2008). Glucose was sterilized separately and aseptically added to the flasks containing the liquid medium, after cooling. The precultures were cultivated in Nutrient Broth medium (0.8% w/v) for 18 h. Then, overnight cultures with OD_{600}=0.3 were inoculated at 1% in enzyme production media (150 mL in 500 mL Erlenmeyer flasks) and incubated at 37 °C for 16, 24, 40, 48, 64 and 72 h in a shaking incubator (150 rpm). At the end of each period, the cultures were centrifuged (6000 rpm, 10 min) and the supernatants were used for determination of proteolytic activity. Bacterial biomass was determined by measuring optical density at 600 nm.

Capabilities of protease production of four strains were examined, and one strain and the best medium were selected for further studies.

Assay of proteolytic activity

Total protease activity was measured using a casein substrate by a modification of the Anson Method (Keay and Wildi 1970). A 1 ml of the culture supernatant was mixed with 1 ml 0.05 M phosphate buffer-0.1 M NaOH (pH 7.0 adjusted with phosphoric acid) containing 2% casein, and incubated for 10 min at 37 °C. The reaction was stopped by adding 2 ml 0.4 M Trichloroacetic acid. After 30 min stand at room temperature, the precipitate was removed by centrifugation and the optical density of the assays was measured at 660 nm. A standard curve was generated using solutions of 0–60 µg/mL tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg/mL tyrosine under the experimental conditions used.

Effect of nutrients on enzyme production

The effects of some nutrients such as carbon, nitrogen sources and some metal salts on enzyme production were investigated. Glucose (0.1% w/v) was replaced in the production medium with maltose, sucrose, fructose, **g**lycerol and starch. Different organic and inorganic nitrogen sources including corn step liquor, skim milk, triptone, soybean, casein, $(NH_4)_2SO_4$ and KNO_3 were tested. These nitrogen sources were used to replace peptone and yeast extract which were the original nitrogen source in growth medium. The culture medium was supplemented with the following metal salts (0.01% w/v): $CaCl_2$ with $LiSO_4$, $BaCI_2$, $MnCI_2$, $CuSO_4$; $MgSO_4$ with $LiSO_4$, $BaCI_2$, $MnCI_2$, $CuSO_4$. Incubation periods were set as 16, 24, 40, 48, 64 and 72 h.

A new medium including the best source of carbon, nitrogen and metal salts for protease production were improved, and bacteria were grown in this new medium.

Partial purification of crude protease and characterization

The crude protease extract was partially purified by ammonium sulphate (75%) precipitation followed by dialysis. The precipitate was collected by centrifugation at 12,500 rpm for 20 min at 4 °C, and dissolved in 0.01 M phosphat buffer (pH 7.0). The solution was dialyzed aganist the same buffer at 4 °C for 8 h with 3 changes of the dialysis buffer, and protein concentration was measured as the method of Lowry et al. (1951) using BSA as standard protein.

After partial purification, the enzyme was characterized with its optimal values of pH (4.0-9.0), temperature (37-80 °C), and their stabilities were presented. The effects of various metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Li^{2+}, Ba^{2+}, Cu^{2+}, Zn^{2+}) on enzyme activity were determined, and enzyme was incubated with metal ions of 5 mM. Relative activities were expressed as a percentage of the activity of the untreated control taken as 100%.

The approximate molecular weight of partially purified enzyme was determined by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli 1970).

RESULTS AND DISCUSSION

Screening and isolation of proteolytic bacteria

Fifty four (54) *Bacillus* sp. strains isolated from soil samples were determined on the basis of morphological and biochemical characteristics. The proteolytic activities of all strains were assayed using skim milk agar and casein agar, and exhibited as diameter of clear zone in mm. Skim milk agar was the best than casein agar

for Qualitative test of protease. Among *Bacillus* sp. strains, six isolates showed high proteolytic activity (18-14 mm), twenty seven isolates showed moderate activity (10-12 mm), fifty five isolates showed weak activity (8-6 mm), and six isolates exhibited very weak activity (4 mm). Four high proteolytic strains (N-3, N-10, N-18, N-40) were checked for Quantitative test of protease in two liquid media. Among tested media, medium of Quadar et al. (2009) was the best than other. In this medium, the maximum protease activity (224 U/ml) was attained after 64 h by *Bacillus* sp. N-40 strain (Table 1). It found that maximum production occured at end of exponential phase.

Table 1. Effect of incubation time on protease production by four *Bacillus* strains during cell growth in medium of Quadar et al. (2009). Values are shown as means of triplicates.

Incubation time (h)	N-3		N-10		N-18		N-40	
	U/ml	OD_{600}	U/ml	OD_{600}	U/ml	OD_{600}	U/ml	OD_{600}
16	50	0.6	24	0.3	52	0.7	83	0.6
24	64	0.6	56	0.5	68	0.9	100	0.7
40	90	0.8	70	0.9	74	1.0	150	0.8
48	99	0.9	85	1.3	90	1.2	182	0.9
64	70	1.0	80	1.1	98	1.1	224	1.1
72	64	1.1	60	0.8	83	0.7	214	1.0

Other three strains showed low production of protease, and *Bacillus* sp. N-40 which is high capability was selected for further studies.

Effect of carbon, nitrogen and metal ions on protease production

Enzyme formation is largely dependent on the condition of growth of the culture and composition of nutrient medium. The present investigation was 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).

The research was focused on the improvement of protease level. Various carbon, nitrogen sources and metal ions were used for the production of protease by *Bacillus* sp. N-40. When glucose in basal medium was replaced by five various sugars (maltose, sucrose, fructose, glycerol, starch), fructose was the best source for protease production (Fig. 1). Fructose (278 U/ml) increased the production of protease by 24% when compared to control (glucose, 224 U/ml). The protease production was affected by carbon sources in the order: fructose >sucrose > maltose> control> starch> glycerol. The increase in enzyme production was parallel to the growth rate (64 h). Glycerol caused reduced growth and protease synthesis.

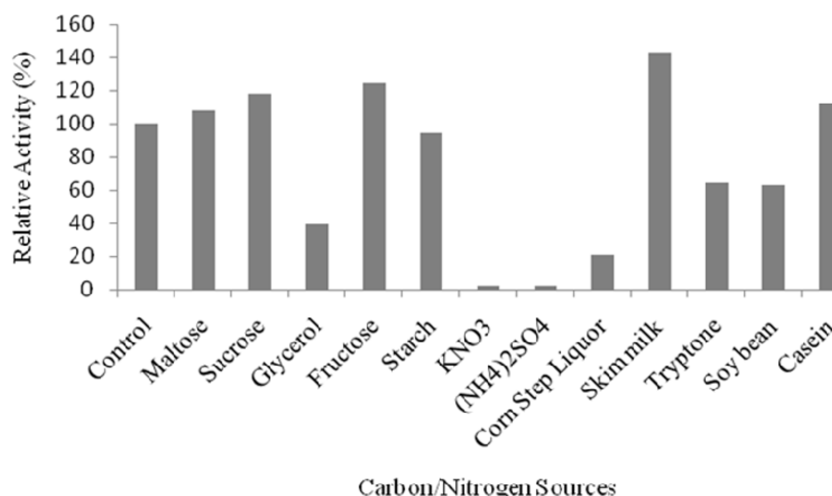

Figure 1. Protease production by *Bacillus* sp. N-40 in various carbon and nitrogen sources.

Reseracherss have been reported different results for the best carbon sources. In a similar study, it showed that fructose was the best carbon source for protease production (Mabrouk et al. 1999; Sangeetha et al. 2008).

On the other hand, Starch, sucrose, and lactose proved appreciably good for the protease production by *Bacillus* sp. K-30 (Naidu and Devi 2005). Alkaline protease production was best in wheat bran, starch, glucose and dextrin by *Bacillus licheniformis* and *Bacillus coagulans* (Asokan and Jayanthi 2010). Lactose was the best carbon source that induced the production of protease by *Bacillus subtilis* (El-Safey and Abdul-Raouf 2004). Madzak et al. (2000) recorded that the sucrose is good substrate for production extracellular proteases. Glucose was found to be the optimum carbon source for protease activity by all the four *Bacillus* isolates followed by sucrose, fructose, maltose, starch and cellulose (Boominadhan et al. 2009). However, other works reported that glucose drastically inhibited protease production (Fukushima et al. 1989; Puri et al. 2002). On the other hand, in this study, starch gave lower growth but higher protease production, but Hadj-Ali et al. (2007) has been reported opposite result. Maltose, starch and cellobiose caused low protease production (Shafee et al. 2005), while starch caused high level of enzyme expression in *Bacillus* species (Johnvesly and Naik 2001).

Nitrogen sources also affected enzyme production. Effects of various organic and inorganic nitrogen sources on production of protease were investigated. Results obtained showed that the best nitrogen source for protease production was skim milk (320 U/ml) at 64 h ,and enzyme yield was 43% compare to control (224 U/ml). Enzyme production in the presence of casein (250 U/ml) reached at 24 h. Maximum enzyme production were skim milk> casein> control> tryptone> soy bean> corn steep liqour (Fig 1). The increase in enzyme production was parallel to the growth rate for all nitrogen sources. Inorganic sources were found to have no effect on protease production, because growth was very low.

The effects of organic and inorganic nitrogen sources on protease production by *Bacillus* sp. have been reported in the literature. Many researchers have been reported that organic nitrogen sources was better suited to *Bacillus* sp. for growth and enzyme production than inorganic sources. It has been reported that peptone, casein, skim milk, yeast extract, favored maximum protease production by *Bacillus* sp (Puri et al. 2002; Sangeetha et al. 2008). Among the organic N sources, the effects of soybean flour, fish peptone, beef peptone and polypeptone were almost the same for *B. pumilus* c172-14 (Feng et al. 2001). The best nitrogen source for protease production was beef extract for *Bacillus* sp. K-30, while yeast extract and tryptone were comparable (Naidu and Devi 2005) . Phadatare et al. (1993) reported the enhancement of protease production in *Conidiobolus coronatus* by organic nitrogen sources like tryptone, peptone and yeast extract. In the present study, addition of inorganic nitrogen sources in the production medium resulted in absence enzyme yield. It has also been reported by Fujiwara and Yamamoto (1987). On the other hand, some inorganic nitrogen sources gave better enzyme production. The best nitrogen source for protease production was $(NH_4)_2SO_4$ (Safey and Abdul-Raouf 2004). Among the various organic and inorganic nitrogen sources, the maximum enzyme activity was obtained with ammonium nitrate followed by ammonium chloride, ammonium citrate and potassium nitrate were used as nitrogen sources (Nascimento and Martins 2004).

The metal ions in media are an important factor that affects enzyme production due to act as inducers. The effects of some metal ions on protease activities were investigated. Firstly, Ca^{2+} and Mg^{2+} ions combined in culture medium (control) was tested along, and showed that enzyme production was very low. Hence, tested metal ions were used combine with each of Ca^{2+} and Mg^{2+}. Addition of Li^{2+}, Ba^{2+}, Mn^{2+} resulted in low production of protease. Cu^{2+} caused inhibition of production. Control (Ca^{2+} and Mg^{2+} combination) was better than tested metal ions (Fig. 2). It has been reported that Ca^{2+} and Mg^{2+} ions was effected on protease production (Feng et al. 2001; Kalaiarasi and Sunitha 2009). It suggested inhibitory effect of Cu^{2+} and Li^{2+} on proteases (Adinarayana et al. 2003).

Both our results and those of other reserachers have been shown that the protease production patways of *Bacillus* strains were very different.

In the present study, the best carbon (fructose), nitrogen (skim milk) and metal (Ca^{2+}+Mg^{2+}) sources for protease production by *Bacillus* sp. N-40 were combined, and obtained a new medium. Production of protease in modified medium was realized. Maximum growth reached at 72 h, while maximum enzyme production (338 U/ml) was at 64 h. It was showed an increase of 51% in protease production when compared with the control (224 U/ml) (Fig. 3).

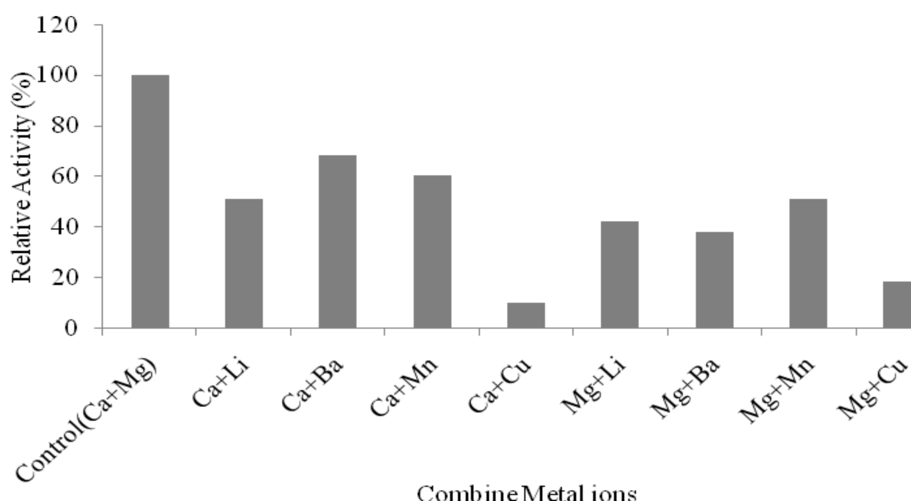

Figure 2. Protease production by *Bacillus* sp. N-40 in the presence of combined metal ions

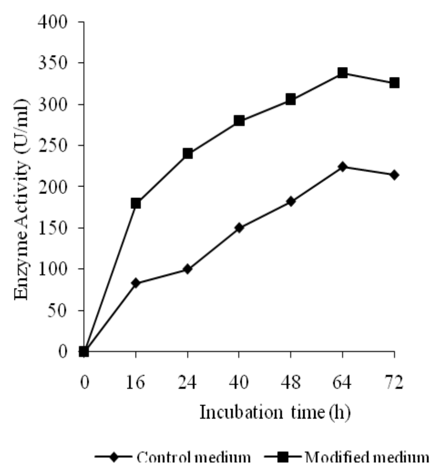

Figure 3. Comparison of protease production by *Bacillus* sp. N-40 in control medium (Quadar et al., 2009) and newly modified medium

Some properties of partial purified protease enzyme

The enzyme was partially purified with a 2.1 fold increase in specific activity with a yield of 30%. Enzymatic properties, such as effect of pH, temperature and metal ions on the activity of the purified protease were performed. The optimum pH of enzyme was determined as pH 7.0 (Fig 4). 100% of enzyme activity was still detectable at pH 7.0 after 4 h at 37 °C. This might be the neutral protease. Because, the optima of most of the neutral protease have been reported in literature supported our finding (Basu et al. 2007; Merhep Dini et al. 2009). It was shown that the enzyme also gave high activity in the alkaline pH range 6.0-9.0 (Fig.4).

The optimum temperature of enzyme was 55°C (Fig. 5). Enzyme activity was stable with temperature within the range of 40ºC to 70ºC as seen in Fig.5. Enzyme was also still active at 80ºC. In literature, optima temperature have been reported between 30-70ºC for *Bacillus* sp. protease (Huang et al. 2006; Morya and Yaday 2010). A similar result, Ammar et al. (2003) reported that, the optimum temperature for thermostable purified protease enzyme was 55 ºC. The thermal stability of protease was examined by pre incubating the enzyme at 55°C for 3h. The protease preserved 77% of its original activity.

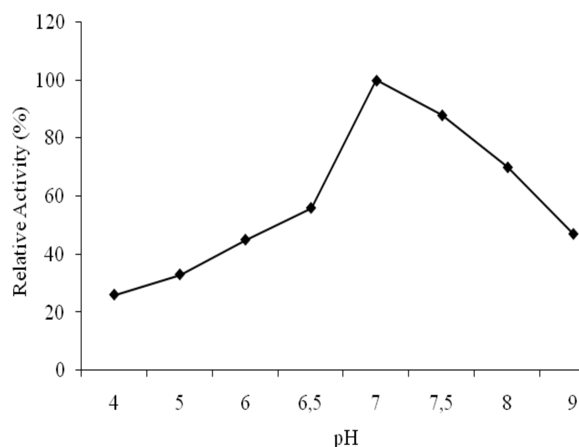

Figure 4. Effect of pH on the protease activity

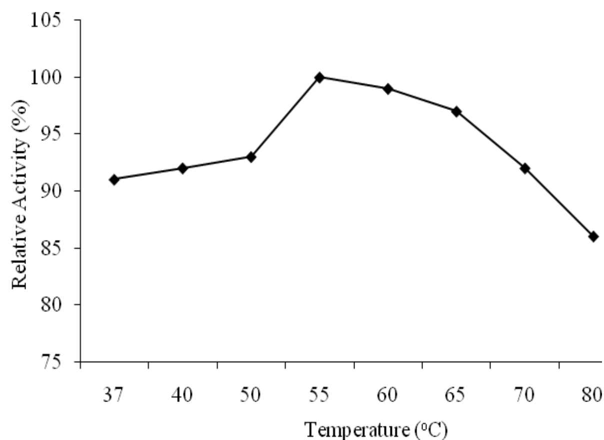

Figure 5. Effect of temperature on the protease activity

Metal ions are known to play a role as cofactors for enzyme activities, and often act as salt or ion bridges between two adjacent amino acid residues. In this study**,** various metal ions such as Li^{2+}, Zn^{2+} Mg^{2+}, Ba^{2+}, Mn^{2+},Ca^{2+},Cu^{2+} at 5 mM concentration were tested for protease activation/inhibition effect, and the results are given in Table 2. Ca^{2+} and Mn^{2+} are found to has activating effect. Enzyme activity increased 28% , 26% in the presence of Mn^{2+} and Ca^{2+} as compared with control, respectively. Li^{2+} had low effect on protease activity. The activity was reduced in the presence of Ba^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}. In the present work, strong activation/inhibition effect of metal ions on protease activity was not observed. Similarly, some investigators has also been reported that protease activity was stimulated by Mn^{2+} and Ca^{2+}(Nascimento and Martins 2004; Jaswal and Kocher 2007). But, the activity was reduced in the presence of Mn^{2+} and Ca^{2+}(Basu et al. 2007; El-Hadj-Ali et al. 2007; Zambare et al. 2007; Merhep Dini et al. 2009) and increased by Zn^{2+} and Ba^{2+} (Jaswal and Kocher 2007). On the other hand, inhibition by Hg^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Ba^{2+}, Zn^{2+} and Li^{2+} was reported by Bhatiya and Jadeja (2010).

Table 2. Effect of various metal ions (5mM) on *Bacillus* sp. N-40 protease activity. (Values are shown as means of triplicates.)

None	100
$CaCI_2$	126
$MgSO_4$	96
$MnCI_2$	128
$BaCI_2$	98
$CuCI_2$	98
$LiSO_4$	102
$ZnSO_4$	70

All the experiments performed independently in triplicate and the results are presented as the mean of three.

This partial purified enzyme was electrophoresed on 10% (w/v) SDS-PAGE and a single band was observed. Using standard protein markers the size of the partially purified enzyme was found to be about 52 kDa (Fig. 6). A varieties of molecular weigth for proteases from other *Bacillus* species had been reported as 49 kDa *Bacillus* sp. HUTBS71 (Akel et al. 2009), 30,9 kDa B*acillus* sp. HS08A (Huang et al. 2006), 75.0 kDa *Bacillus sp.* S17110 (Jung et al. 2007).

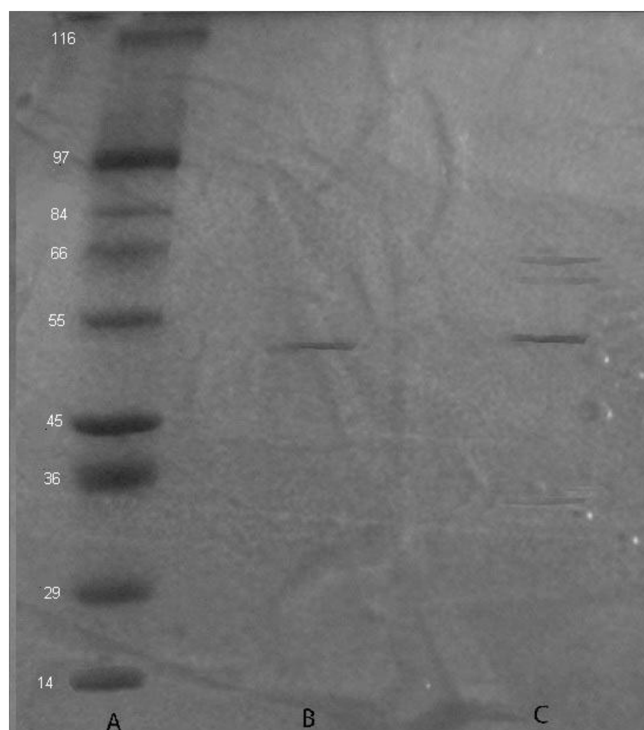

Figure 6. SDS-PAGE showing the molecular weight of protease enzyme produced by *Bacillus* sp. N-40 (52 kDa). A: S8445 SigmaMarker, B: Dialysate, C: Crude extract

CONCLUSIONS

A new strain of *Bacillus* was found to be a potential producer of protease enzyme. Studies on *Bacillus* sp. N-40 showed that nutritional factors include of sources of carbon, nitrogen and metal ions can inflence production of protease. Enzyme was partially purified, and characterized. This strain might be suitable for many industrial applications. Further experiments to enhanced enzyme production for commercialized process is needed.

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