Original article

Optimization and Production of alkaline Proteases from *Bacillus subtilis MMS15* isolate

Mohammed Salih Osman*[1],Ayman Salih Omer Idris[2] , Dayanandan Anandan[3], Amar Mohamed Ismail[2], Adil A. El-Hussein*[4]

[1] *Department of Food Industries Research, Industrial Research & Consultancy Center, Khartoum Bahri, Sudan*
[2]*Department of Biochemistry & Molecular Biology, Al- Neelain University, Khartoum, PO Box 12702, Sudan.*
[3]*International Research Center, Sathyabama University, Chennai, India* [4] *Department of Botany, University of Khartoum – Sudan.*

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Abstract

Protease enzyme is most interest enzyme in industrial biotechnology, which capable of degrading the protein into amino acids and small peptide. They account for more than 50% of the total enzyme market. We report the production of alkaline protease enzyme from *Bacillus subtilis* isolate *MMS15***.**Different conditions including pH values, incubation temperatures and periods, carbon and nitrogen sources and inoculum sizes were applied for the protease production optimization. The maximum activity for protease was recorded at alkaline pH 10.0 at 37 ^{0}C with an incubation period of 48 hours and inoculum size of 5%. The best Carbon and nitrogen source for protease production were found to be glucose and yeast extract plus pepton, respectively. The results showed that *B. subtilis MMS15* is a potential producer of extracellular proteases under these conditions, and thus can be a promising candidate for industrial applications.

*__Corresponding author__: mhommed23@yahoo.com

Introduction

Microorganisms are known to play important role in biotechnology for production of intracellular and extracellular enzymes and products on an industrial scale (Da Silva *et al*., 2016; Gupta *et al.*, 2002) selected organism are grown under optimized condition to achieve high production of enzymes and products (Rathakrishnan *et al.*, 2012).

Proteases (EC 3:4, 11-19, 20-24, 99) constitute a large and complex group of proteolytic enzymes (Jisha *et al.* 2013)**.** These enzymes carry out proteolysis by hydrolysis of the peptide bond that exists between two amino acids of a polypeptide chain (Singhal *et al.* 2012). Although proteases can be produced from different sources such as plants, animals and microorganisms, the available proteases in the market are mostly derived from microbial sources. Proteases have a wide range of applications in biodetergents (Amara *et al.*, 2009), food (Rao et al., 1998), pharmaceutics (Nishina *et al.*, 1992), Leather industries (Zhao *et al.*, 2012), silver recovery (Joshi and Satyanarayana, 2013) and bioremediation processes (Seifzadeh *et al.*, 2008). Many species of the genus Bacillus produce large amounts of protease extracellularly during the post-exponential and stationary growth phases into the culture medium (Lin *et al.*, 2015). This is why they are involved in protease production, example include: *Bacillus clausii* (Christiansen and Nielsen, 2002; Joo and Chang, 2006; Oskouie *et al.*, 2008), *Bacillus cereus* (Uyar *et al.*,2011), *Bacillus circulans* (Benkiar *et al.*, 2013*), Bacillus lehensis* (Joshi and Satyanarayana, 2013),

Bacillus licheniformis (Lin *et al.,* 2015), *Bacillus megaterium* (Asker *et al.,* 2013), *Bacillus mojavensis* (Haddar *et al.,* 2009), *Bacillus pumilus* (Jaouadi *et al.,* 2008), *Bacillus stearothermophilus* (Sookkheo *et al.,* 2000), and *Bacillus subtilis* (Maruthiah *et al.,* 2013).

Bacilus subtilis is one of the most important bacteria for the production of specific chemical products and industrial enzymes and also considered a main source of amylase and protease enzymes. (Gupta *et al.,* 2002).

With the fast grow of biotechnology, there has been an increase the demand for enzymes with specific properties and characterization. So we need to explore difference sources of proteases with high productivity. Production of proteases depends on many factors such as nutrient and cultural conditions for the selected organism. So it is necessary to know the suitable nutrients and optimum cultural conditions to obtain high production of protease (Sana *et al.,* 2006).

Therefore, it's highly intriguing for biotechnologist to find bacterial isolates with high potentiality for proteases productions (Tambekar *et al.* 2011). The present study aimed to optimize various growth parameters for alkaline protease production by *Bacillus sp. MMS15* locally isolated under submerged fermentation conditions.

Material and methods

Chemicals

All chemicals used in this study with high purity (99%) were purchased from Hi-Media Laboratories, Merck (Mumbai, India) and Sigma (U.S.A).

Bacterial strain

Bacillus subtilis MMS15 (GenBank accession No. KT933118) isolated from soil samples collected from Khartoum state, Sudan was used in this study (data not published).

Production by *B. subtilis MMS15* isolate Protease

Fifty milliliter of pre-culture medium consisting of 0.8 % nutrient broth (Peptone, 0.5%; yeast extract, 0.5%; pH 7.5) was transferred to the each cotton plugged 250 ml Erlenmeyer flasks and sterilized in an autoclave for 15 min at 15 lb/inch^2 (121ºC). After cooling at room temperature, the flasks were inoculated aseptically with a loopful of bacteria from 24 h old slant. The flasks were then rotated at 100 rpm in a shaking incubator at 37 °C for 24 hrs. Then 5 ml of overnight culture of *Bacillus subtilis MMS15* was used to inoculate 50 ml Erlenmyer flask containing 45 ml aliquots of liquid production medium consisting of 1% (w/v) glucose, 0.5% (w/v) peptone and yeast extract, 1.0 % (w/v) KH_2PO_4, 0.2% (w/v) $MgSO_4.7H_2O$, pH 9.0 and maintained at 50 °C for 24 h in a shaking incubator operating at 150 rpm. The pH of the medium was adjusted by 1N NaOH or 1N HCl. After an incubation period of 24 h the whole fermentation broth was centrifuged at 10,000 rpm at 4 °C for 15 min and clear supernatant was recovered as crude enzyme.

Protease assay

Protease activity was assayed according to Beg and Gupta (2003), with slight modifications. The reaction mixture contained 2 ml of 1% casein and 1 ml of appropriately diluted enzyme in the presence of 50 mM Glycine/NaOH buffer (pH 10.0). The reactants were incubated at 37 °C for 10 min and the reaction was stopped by adding 3 ml of 10% trichloroacetic acid (TCA). A suitable blank of deactivated enzyme was used. After incubating at 37 °C for 30 min, both test and blank solutions were centrifuged at 10,000 rpm for 10 min. To 1 ml supernatant, 2.5 ml of 50 mM Na_2CO_3 and 0.5 ml of 0.5 M Folin-ciocalteau reagent were added, then the reaction mixture was incubated at room temperature for 30 min and absorbance was measured at 660 nm. One unit (U) of proteolytic enzyme activity was defined as the amount of enzyme that liberate 1 µg tyrosine per ml per minute under specified assay conditions.

Optimization of enzyme production at different fermentation conditions

Effect of different pH values

The effect of pH on protease production by *B. subtilis MMS15* was investigated by growing each isolates in the assay medium using different levels of pH viz 5, 7, 8, 9, 10, 11 and 12 with required concentrations of 1N NaOH and 1N HCl. Protease concentrations were determined using protease assay as mentioned earlier (Materials and Methods).

Effect of incubation temperatures

The effect of temperature on protease production by the bacterium was determined by growing it in the assay medium

(pH 10) at different temperatures (25, 30, 37, 40, 45, 50, 55 °C) for 48 h at 150 rpm in the shaker incubator. The protease assay was carried out to determine the concentration of the enzyme.

Effect of Incubation period

The isolate was inoculated into the assay medium (pH 10) and incubated at 37 °C and 150 rpm. The protease activity was monitored at intervals of 12, 24, 36, 48, 72, 96 and 144 h.

Effect of inoculum size

Different inoculum sizes (2%, 4%, 5%, 6%, 8% 10%, and 12%) were used to inoculate the production medium and after 48 h of incubation enzyme activity was assayed.

Effect of different carbon sources

The effect of various simple and complex carbon sources on the production of protease by bacterium was studied by replacing glucose with 1% of each of carbon sources under study; sucrose, lactose, maltose, mannose, mannitol and starch. The medium pH, temperature and incubation time was set at 10.0, 37 °C and 48 h, respectively, under shaking conditions at 150 rpm.

Effect of different nitrogen sources

Various organic nitrogen sources including peptone, yeast extract, casein, beef extract, gelatin and inorganic nitrogen source such as $NaNO_3$ and KNO_3 were each used to replace the yeast extract and peptone in the protease production medium.

Results and Discussion

Effect of different pH values

38% and 14% increments in protease production were recorded when the pH was increased from 7 to 8, respectively (Fig.1). However, when the pH was increased from 8 to 9 an increase in protease production of 145% was observed. The optimum protease activity (160 U/ml) was recorded at pH value of 10 (>145%). This pH value has been previously reported to have maximum protease production by B. circulans MTCC 7942 (Patil & Chaudhari 2013), B. licheniformis Bl8 (Lakshmi & Parasad 2013). However, some others investigators have reported pH value of 8, 8.5, 9 and 11 for optimal protease production by B. coagulans PSB-07 (Olajuyigbe & Ehiosun 2013), B. amovivorus (Sharmin et al. 2005), B. subtilis RSKK96 (Akcan & Uyar 2010), B. cohnii APT (Tekin et al. 2012), respectively.

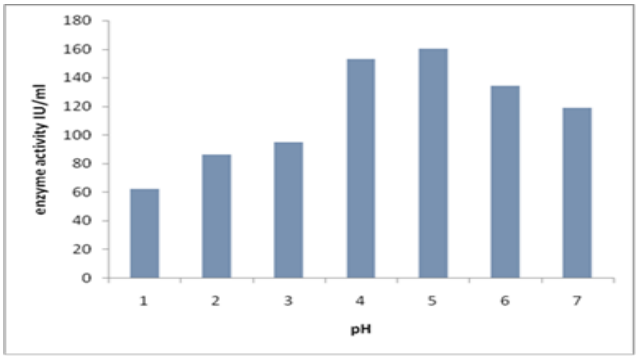

Figure (1) Enzyme production by *B. subtilis* at different pH

Effect of incubation temperature

The highest protease production of 179.34 U/ml in glucose medium by *B. subtilis* MMS15 was observed at 37 °C, whereas the lowest production of 100.12 U/ml was recorded at 55 °C (figure. 2). This is in accordance with previous studies which reported that 37 °C is an optimal temperature for protease production by *B. subtilis* and *B. cereus* (AL-Abdalall & Al-khaldi 2016; Lakshmi *et al.* 2013). However, 30 °C was found to be optimal for protease production by *Bacillus* sp. MIG (Mohapatra *et al.* 2003). Many investigators studied the relationship between different temperatures and protease production and reported that protease production depends on the type of organism used, the fermentation conditions and different of enzyme (El-Safey & Abdul-Raouf 2004).

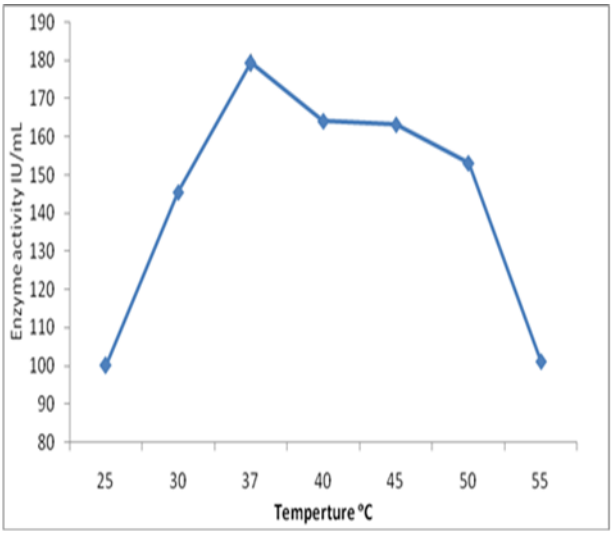

Figure 2: Showed the effect of temperature on protease production by *B. Subtlis*

Effect of incubation period

The maximum (170.34 U/ml) protease production was observed after 48 h of incubation (Table. 1). Previous studies showed that 48 h of incubation for *B. subtilis* was a suitable incubation time for maximum production of protease (Jadhav *et al.* 2013), and also a maximum production of proteases at 48 to 72 h of incubation was reported (Hoshino *et al.* 1997). Similar results were also observed for *B. subtilis* AKRS3 (Krishnan Ravishankar *et al.* 2012). The duration needed for incubation is generally dictated by the properties of the strain such as its growth rate and enzyme production pattern (Jadhay *et al.* 2013).

Table 1: showed the effect of Incubation time on the enzyme production.

Incubation time (per hours)	U/ ml
12	113.2
24	159.11
48	170.34
72	145.12
96	96.22
120	84
144	76

Effect of inoculum sizes

The inoculums size have been reported to affect the production of microbial enzymes

Inoculum size concertedly interacts with the available materials that enhance the protease production. Maximum protease production (183.41 U/ml) was recorded when 5% inoculum size was used (figure. 3). This result is consistent with the result reported previously for *B. subtilis* (Abusham *et al.* 2009). Sinha and Satyanarayana (1991) have studied the alkaline protease production *by B. licheniformis* N3 and reported high yield with the use of inoculum at 0.5-8% level. On the other hand, Sen and Satynarayana (1993) 2% inoculum size appeared to be favorable for protease production by *B. licheniformis* S40 and 4% inoculum size for *B. coagulans* PB77 (Gajju *et al.* 1996).

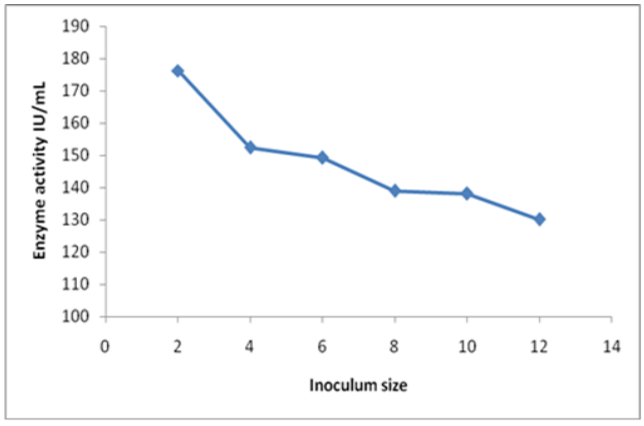

Figure 3: showed the effect of inoculums size on the enzyme production

Effect of carbon sources

Glucose was found to be the best carbon source for extracellular protease production by *B. subtillus MM15* when compared with other carbon sources (Fig. 4). Similar results were also reported previously Beg *et al.* (2003) for *B. mojavensis*, Nisha and Divakarna (2014) for *B. subtillus* and Mehrotra *et al.* (1999) for *Bacillus sp*. One percent glucose has been reported to be the best for the production of alkaline proteases from *B. subtilis* AG-1 (Ghafoor & Hasnain, 2009 and *B. licheniformis* (El Enshasy *et al.* 2008). However, Mabrouk *et al.* (1999) reported that the highest yield of alkaline protease was achieved when 4% lactose plus 15% glucose were used.

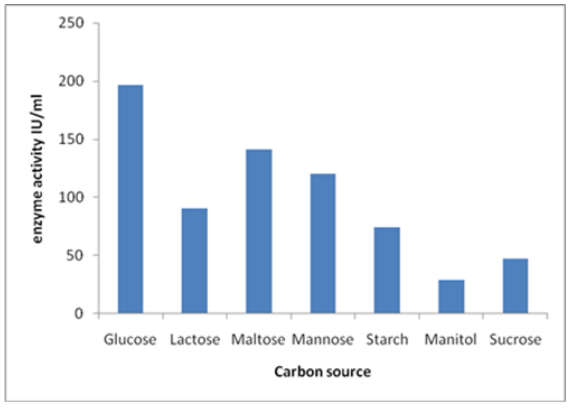

Figure 4: showed the effect of different carbon source on the enzyme production

Effect of nitrogen sources

Different organic and inorganic nitrogen sources i.e. peptone, yeast extract, casein, beef extract, gelatin and inorganic nitrogen source such as NaNO3 and KNO3 were each used to replace the yeast extract and pepton in the protease production medium at 0.5 % (w/v) concentration. The highest production of 196.33 U/ml (Fig. 5) was obtained with the combination of yeast extract plus peptone followed by beef extract with 144.41 U/ml. Similar results were reported showing that yeast extract and peptone as optimal nitrogen sources for protease production (Atalo & Gashe 1993; Kumar 2003). Intriguingly, yeast extract only has increased the protease production by *Streptomyces sp. CN902* (Lazim *et al.* 2009). On the other hand, Kumar and Vats (2010) reported that Peptone was optimum nitrogen source for protease production from a *B. subtilis* MTCC 9102.

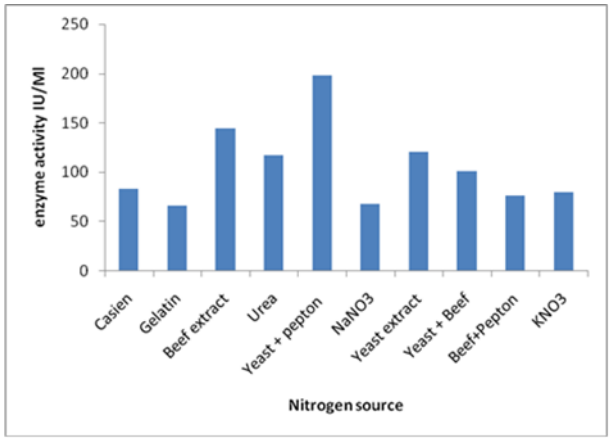

Figure 5: Effect of nitrogen source on the enzyme production

Conclusions

- Optimization of various parameters shows that glucose, yeast extract and peptone at pH 10 and at 37 °C were considered as optimum conditions for maximum alkaline protease production by *B. subtilis MM15* strain.
- *B. subtilis MMS15* isolate has a high potentiality and effectiveness as an important biotechnological tool for various industrial applications.
- Further studies were needed to elaborate he enzyme kinetics and its production screening especially for large scales are highly demanded.

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