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Full Length Research Paper

Some properties of extracellular protease from *Bacillus licheniformis* LBBL-11 isolated from "iru", a traditionally fermented African locust bean condiment

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Twelve strains of *Bacillus licheniformis* isolated from traditionally fermented African locust bean (*iru*) were evaluated in respect to production of protease on skim milk agar. *B. licheniformis* LBBL-11 exhibited the highest proteolytic activity with a diameter of clear zone measuring 35.0 mm. Production of protease from *B. licheniformis* LBBL-11 was further studied by growing the strain on nutrient broth. Maximum protease production was 18.4 U/ml at 48 hour of growth which coincided with the end of exponential phase. The protease from this *Bacillus sp* had optimum pH of 8.0 and was stable over a wide pH range of 5.0 - 11.0. The optimum temperature for the protease activity was 60°C. The enzyme was 95% stable at 60°C after 60 min of incubation. These properties indicate possible application of *B. licheniformis* LBBL-11 as potential starter culture for the fermentation of African locust bean under controlled conditions of temperature and pH.

Key words: Fermented locust bean*, iru,* protease, thermostable, *Bacillus licheniformis*.

INTRODUCTION

African locust bean (*Parkia biglobosa)* is rich in protein and usually fermented to a tasty food condiment called '*iru*' which is used as a flavor intensifier for soups and stews in Nigeria (Odunfa, 1985; Allagheny et al., 1996). This is highly consumed in developing and under developed countries where there is high cost of animal protein. However, the traditional fermentation techniques are often characterized by the use of simple, non-sterile equipment, chance of natural inoculum and unregulated conditions (Nout, 1985) that lack control of microbial development which can allow pathogens to survive or produce toxins in such products. Hence for the safety of consumers, there is strong need for pure starter cultures that can be used under aseptic conditions, controlled pH and relatively high temperature. This makes it very difficult for other microorganisms that might spoil the product to grow.

Proteolysis is the main metabolic activity during the fermentation of African locust bean which also contributes to the development of texture and flavour of fermented products (Odunfa, 1985; Allagheny et al., 1996; Ouba et al., 2003). The desired state of fermentation of the condiments is indicated by the characteristic ammonia odour produced as a result of breakdown of amino acids during fermentation.

There have been reports on use of *Bacillus subtilis* as starter culture for fermentation of African locust bean (Odunfa and Oyewole, 1986; Ouoba et al., 2003; Terlabie et al., 2006) but none has addressed the problem of obtaining a microorganism that can be used under highly regulated controlled conditions of temperature and pH for starter culture. In this study we examined protease production from *B. licheniformis* strains which formed 34% of the identified *Bacillus* isolates from traditionally fermented African locust bean and determined some fundamental properties of the protease.

MATERIALS AND METHODS

Isolation of microorganisms

Traditionally fermented African locust bean was purchased from three different locations in Akure, South-west of Nigeria. 0.1 g samples were homogenized with 5.0 ml of sterile water and agita-

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ted for 30 min on a shaker at 75°C. The suspension was then diluted in serial 10-fold dilution steps up to 10^{-7}. One ml of each dilution was poured into Petri dishes containing nutrient agar. The inoculated Petri dishes were then incubated at 37°C for 24 h.

Identification of the proteolytic isolates

The proteolytic bacterial isolates were identified by conventional microbiological methods (Holt et al., 1994; Beckley et al., 1984). The cells were characterized based on cellular morphology of the cell, gram characteristics, motility and biochemical tests such as citrate utilization, oxidase, urease, gelatin hydrolysis, catalase, Voges- Proskaver and indole tests, fermentation of D-Glucose, D-Arabinose, D- Lactose, D-Mannitol, D-Galactose and D-Maltose. The identified strains of *Bacillus licheniformis* were labeled LBBL 01 to LBBL-12 and were maintained on nutrient agar slants at 4°C.

Screening of Bacillus licheniformis isolates

B. licheniformis isolates were screened for protease production on skim milk agar plates containing peptone (0.1% w/v), NaCl (0.5% w/v), agar (2.0% w/v) and skim milk (10% v/v). The inoculated plates were then incubated at 37°C for 24 h and observed for zones of clearance which indicate proteolytic activities.

Cultivation of microorganisms and protease production

B. licheniformis LBBL-11 which exhibited the highest proteolytic activity was cultivated in 50 ml nutrient broth and incubated at 37°C in a shaking incubator (150 rpm) for 72 h in triplicate. Protease production and cell population were monitored at every 6 h interval. The optical density of the broth at 660 nm was measured in a Biochrom uv/visible spectrophotometer to obtain the cell population. At every 6 hour interval, 10 ml of the fermentation broth was centrifuged at 10,000 rpm for 15 min at 4°C and clear supernatant was used as crude enzyme preparation. Protease production over the 72 h cultivation period was determined through assay of protease activity in the crude enzyme extracts. Protease activity was determined by the method of Sarath et al. (1989) using azocasein as substrate. 500 µL of 0.5% azocasein in Tris-HCl buffer pH 8.0 was incubated with 100 µL enzyme solution for 60 min at 37°C. Reaction was stopped by adding 500 µL of 15% Trichloroacetic acid (TCA) with shaking. This was left for 15 min and centrifuged at 4°C for 15 min at 3000 rpm. 1 ml of supernatant was added to 1 ml of 1 M NaOH and absorbance was read at 440 nm.One unit (U) of protease activity was defined as micromole of substrate converted per minute under standard assay conditions.

Effects of pH on protease activity and stability

The effect of pH on protease from *B. licheniformis* LBBL-11 was determined by assaying the enzyme activity at different pH values ranging from 5.0 to 11.0. The pH was adjusted using the 0.05 M of the following buffer solutions: citrate-phosphate (pH 5.0 - 6.0), Tris-HCl (pH 7.0-8.0) and glycine-NaOH (pH 9.0 – 11.0). The effect of pH on enzyme stability was determined by pre-incubating the protease without substrate at different pH values (5.0 – 11.0) using relevant buffers for 1 h at 37°C after which the residual protease activity was determined.

Effects of temperature on protease activity and stability

The effect of temperature on protease activity was determined by incubating the reaction mixture (pH 8.0) for 1 h at different temperatures ranging from 30 - 80°C. The activity of the protease was then measured according to the standard assay procedure. Thermostability of protease was determined by incubating protease at temperatures ranging from 30 – 80°C without substrate for 1 h in a constant temperature water bath, after which the residual activity was determined.

RESULTS AND DISCUSSION

Screening of *Bacillus* isolates

We have isolated 35 *Bacillus* strains (12 *B. licheniformis* 34%; 21 *B. subtilis* 60 % and 2 *B. cereus* 6%) from traditionally fermented African locust bean sample suspension with the aim of obtaining a novel strain which produces high yield of thermostable alkaline protease. These findings show that *B. licheniformis* and *B. subtilis* are the major *Bacillus* species involved in the fermentation of the African locust beans. There have been reports on the different Bacillus sp isolated from fermented African legumes (Odunfa, 1981; Omafuvbe et al., 1999). For instance in Republic of Benin, Azokpota et al. (2006) reported *B. subtilis* and *B. licheniformis* as predominant *Bacillus sp.* from three fermented locust bean condiments. The *B. licheniformis* strains are of great interest in this study because there is scarcity of reports on protease from this *Bacillus sp* and its use as starter culture for the fermentation of African locust bean.

All the isolated *B. licheniformis* strains exhibited vivid zones of clearance on 10 % skim milk agar. *B. licheniformis* LBBL-11 showed highest proteolytic activity with an average clear zone diameter of 35 mm amongst all twelve *B. licheniformis* strains tested (Figure 1). Consequently, *B. licheniformis* LBBL-11 was selected for further studies.

Cultivation of microorganisms and protease production

Protease production of *B. licheniformis* LBBL-11 grown in 50 ml nutrient broth over a 72 hour period increased exponentially from 18 hour reaching the maximum yield of 18.4 U/ ml at the 48 hour of cultivation while it started decreasing after 48 hour (Figure 2). It was interesting to discover same trend with growth of *B. licheniformis* LBBL-11 (Figure 2) though the growth was almost stationary between 42 and 48 hours. This suggests that production of protease by this *Bacillus sp*. is dependent on cell growth. Similar reports were obtained from studies on *B. subtilis* (Amin et al., 2004; Omafuvbe, 2006).

Effects of pH on protease activity and stability

Figure 3 shows effects of pH on the activity and stability of the protease produced from *B. licheniformis* LBBL-11. The protease was active over the entire pH range of 5.0 to 11.0 studied. The highest protease activity (100%) was found at pH 8.0 while at pH 5.0, about 69% of the maxi-

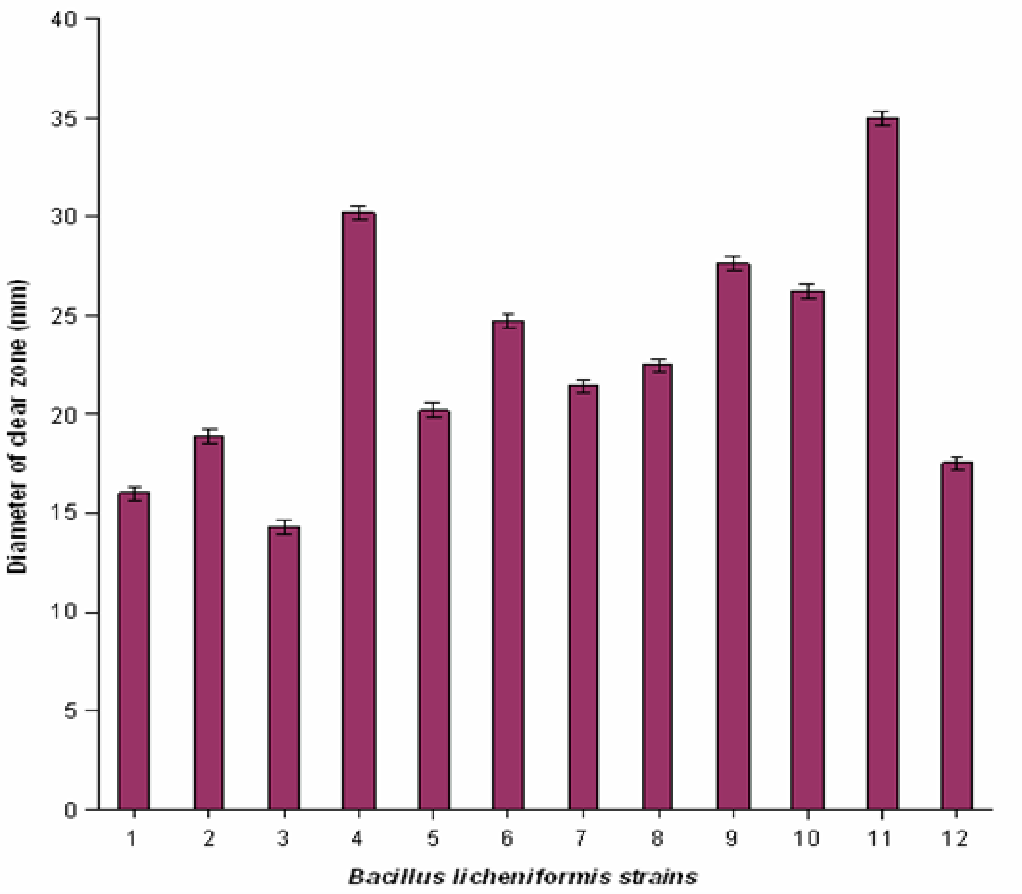

Figure 1. Proteolytic activity of *Bacillus licheniformis* strains on skim milk agar bars represents S.D.

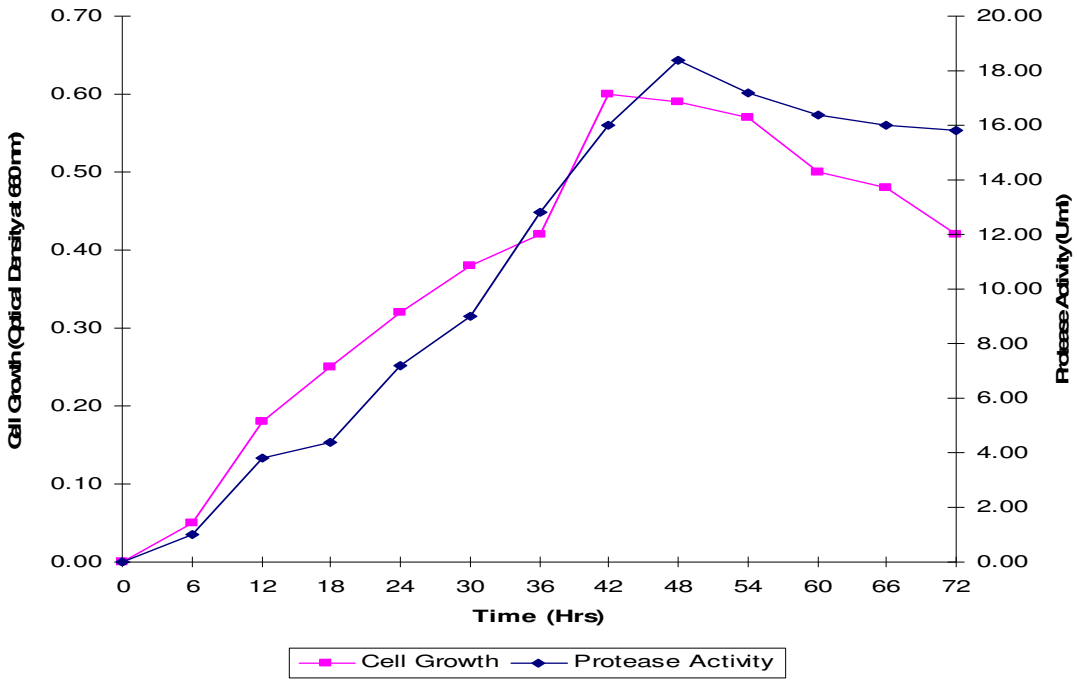

Figure 2. Growth kinetics of *Bacillus licheniformis* LBBL-11 and protease production.

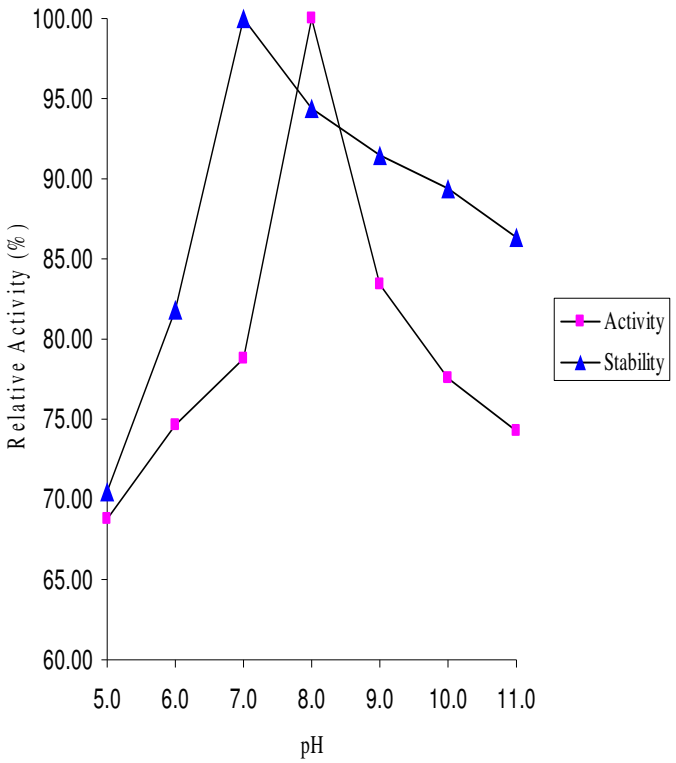

Figure 3. Effect of pH on the Protease Activity and Stability produced from *Bacillus licheniformis* LBBL-11

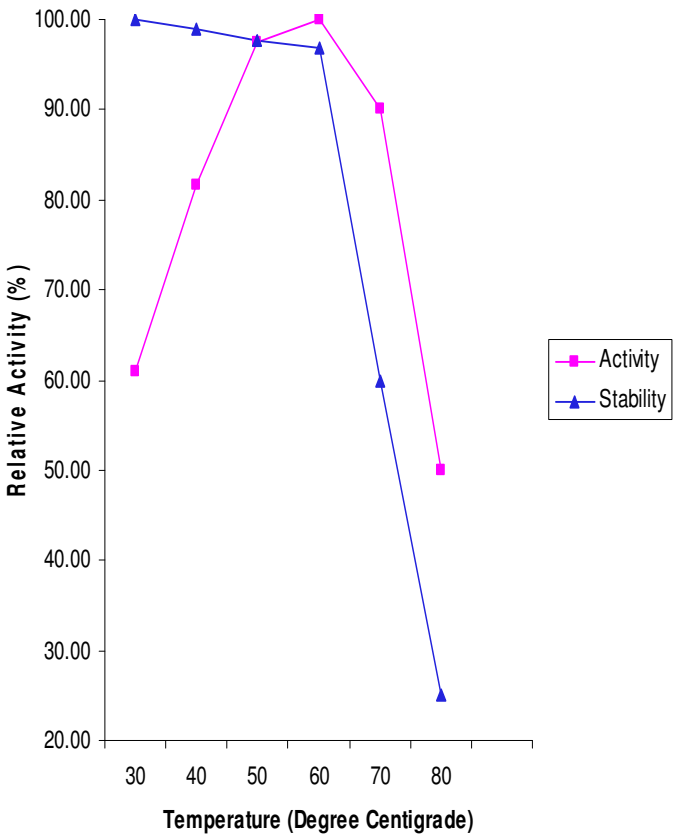

Figure 4. Effect of temperature on the protease activity and stability produced from *Bacillus licheniformis* LBBL-11

maximum enzyme activity was observed, increasing to 75 and 79% at pH 6.0 and 7.0. The protease retained 83, 78 and 74% of the maximum enzyme activity at pH 9.0, 10.0 and 11.0. The effect of pH on stability of this alkaline protease under study showed that the enzyme had optimum pH for stability at 7.0 while it retained 86% of its activity at pH 11.0 and 70% at pH 5.0 (Figure 3). This shows a high pH tolerance by the protease produced from this *Bacillus sp* under study in terms of activity and stability. A report by Chantawannakul et al. (2002) showed the protease from *B. subtilis* isolated from fermented soybean in Northern Thailand which had an optimum pH of 6.5. The ability of this protease from *B. licheniformis* LBBL-11 to maintain high activity over the entire pH studied indicates that this *Bacillus sp* has the potential for use as a starter culture for fermentation of African locust bean, under varied conditions of pH.

Effects of temperature on protease activity and stability

The optimum temperature for the activity of protease from *B. licheniformis* LBBL-11 was 60°C (Figure 4). Protease activity increased with temperature within the range of 30°C to 60°C. A reduction in enzyme activity was observed at temperatures above 60°C. Surprisingly, the protease still retained 50% of its optimal activity at 80°C

The study on the thermostability of protease showed that the enzyme was 95% stable at 60°C while 40 and 75% of the original activities were lost respectively at 70 and 80°C (Figure 4). Our results show that this protease is thermostable. This demonstrated thermotolerance of the protease is of particular interest especially when compared with some reports on protease from other *Bacillus sp* which had relatively lower thermotolerance. For instance, protease obtained from culture of the wild *Bacillus* strain isolated from coffee bean was stable up to 50℃ (Dias et al., 2008) while that reported by Chantawannakul et al. (2002) from *B. subtilis* isolated from fermented soybean had optimum temperature of 45°C.

The unique properties exhibited by the protease from B. licheniformis LBBL-11 under our study show that this *Bacillus sp* is a good producer of thermostable alkaline protease and can be a potential starter culture for the fermentation of African locust bean under controlled conditions of pH and temperature.

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