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Effect of calcium carbonate nanoparticles on alkaline protease production from *Streptomyces spororaveus*

Waleed Mohamed Hussain Abdulkhair[1*] and Mousa Abdullah Alghuthaymi[2]

[1]General Department of Basic Medical Sciences, Microbiology Department, National Organization for Drug Control and Research (NODCAR), Elmansoria street, P.C. 12553, Giza, Egypt.
[2]Biology Department, Science and Humanities College, Shaqra University, Saudi Arabia.
Email: waleed_hamada@yahoo.com

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ABSTRACT

Aims: This study aims to detect the effect of nano-sized calcium carbonate as an ingredient in growth medium on the production of alkaline protease by *Streptomyces spororaveus*. The proportional relationship between highly production of alkaline protease and calcium carbonate nanoparticles emphasizes the unique and super properties of nanotechnology that applied in all field.
Methodology and results: The high production of protease from *S. spororaveus* accompanied with presence of calcium carbonate nanoparticles as one of growth medium's constituents. Both qualitative and quantitative tests for proteolytic activity proved this fact. Agar-well diffusion method revealed that, the proteolytic activity with calcium carbonate nanoparticles (45 mm) is higher than that with calcium carbonate (30 mm). Calcium carbonate nanoparticles led to 150 µg/mL of protease, while calcium carbonate led to 65 µg/mL only. The crude protease was purified by ammonium sulphate precipitation and gel filtration column chromatography using Sephadex G-100. The purified protease was separated by SDS-PAGE as a single band at 30 kDa. The highest proteolytic activity was obtained at pH 8.5 and 45 °C as optimum environmental conditions. The purified protease has inhibited the growth of *Candida albicans* ATCC-10231 and *Aspergillus brasiliensis* ATCC-16404 at 8 mm and 10 mm of inhibition zone respectively.
Conclusion, significance and impact of study: Calcium carbonate nanoparticles in the composition of starch nitrate broth is good stimulus for highly proteolytic activity of *S. spororaveus*. Shake-flask fermentation method proved that, the concerned protease is an alkaline and thermostable up to 70 °C. However, the best pH and temperature values are 8.5 and 45 °C, respectively. This study can be applied to manufacture a modified starch nitrate broth medium for highly production of proteases from *Streptomyces* bacteria.

Keywords: Streptomyces, nanotechnology, enzymology, protein

INTRODUCTION

The genus *Streptomyces* is a powerful producer for different enzymes including proteases which are widely used in the industry (Gilbert *et al*., 1995). Proteases are produced by various species of *Streptomyces* bacteria, such as *S. griseus* (Hadeer, 1999), *S. rimosus* (Yang and Wang, 1999), *S. thermovulgaris* (Yeoman and Edwards, 1994), and *S. griseorubens* E44G (Abdulaziz *et al*., 2015). The proteolytic activity of *S. griseorubens* E44G is the main antifungal mechanism against *Rhizoctonia solani* (Abdulaziz *et al*., 2015). Although microbial proteases are well known as antimicrobial agents, they are widely used in different industries (Deng *et al*., 2010) such as detergents, textiles, and pharmaceuticals industries (Leisola *et al*., 1996; Showell, 1999). Proteases are divided into three types according to their optimum pH value; neutral, acidic and alkaline proteases. Each type of them is subdivided into six sub-types according to the functional groups attached with the active sites; aspartic, glutamic, metallo, cysteine, serine, and threonine proteases. Serine proteases have highly proteolytic activity in particular against recalcitrant animal proteins such as collagen and keratin (Uesugi *et al*., 2011). The structure of alkaline serine proteases has three catalytic residues in their active sites; aspartate, histidine, and serine which called a catalytic triad (Gupta *et al*., 2002). Amongst microbial proteases, alkaline proteases are widely used in different industries (Agrawal *et al*., 2012). The purified form of enzymes must be used in the industry due to high specific activity to be more convenient and effective (Adinarayana, 2003).

Carbon dioxide is a precursor of carbonates during mineralization process, in which carbon dioxide is

*Corresponding author

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captured from different industrial sources and then converted to carbonates, which can be transformed to nanoparticles under certain conditions (Lacroix and Larachi, 2008). Calcium carbonate nanoparticles are applied in several industries such as paper and plastic industries and controlling of rheology due to their super and unique properties (Chen *et al*., 1989; Haruta *et al*., 2003). The size of calcium carbonate particles controls their applications. Calcium carbonate is a polymorphic salt, where it has three thermodynamic stable forms, which arranged descendingly as calcite, aragonite, and vaterite (Sawada and Ohtaki, 1998). Although calcium carbonate nanoparticles can be prepared by different methods such as precipitation or wet carbonation (Xiang *et al*., 2004), use of emulsion membranes (Hu *et al*., 2004), and high gravity reactive precipitation (Chen *et al*., 2000), precipitation method is a more common due to its simplicity and efficacy. There are two precipitation methods; the first one depends on the reaction of calcium hydroxide with carbon dioxide to form calcium carbonate as a precipitated salt, the second method depends on either the reaction of sodium carbonate with calcium chloride or sodium carbonate with calcium nitrate to form calcium carbonate. In precipitation method, the crystallization form and particle size distribution of calcium carbonate mainly depend on the supersaturation level and ionic ratio of calcium and carbonate ions (Chakraborty and Bhatia, 1996). Calcium carbonate can be used as carbon source to support the growth and for selective isolation of actinomycetes. Tsao *et al*. (1960) stated that, good growth of actinomycetes was obtained when hydrated soil was mixed with calcium carbonate and then incubated at 28 °C. Bhuvaneshwari *et al*. (2016) reported that, calcium ions increase the hydrolytic and alkaline activity of protease at optimum temperature. This study aims to improve the hydrolytic activity and thermostability of alkaline-serine protease produced by *Streptomyces* bacteria using nano-sized calcium carbonate containing starch nitrate broth during submerged fermentation.

MATERIALS AND METHODS

Test microorganisms

The strain of *S. spororaveus* which isolated and identified by Abdulaziz and Abdulkhair (2012) was used in this study. On the other hand, *C. albicans* ATCC-10231 and *A. brasiliensis* ATCC-16404 were used as test fungi.

Preparation of calcium carbonate nanoparticles

Calcium carbonate nanoparticles were prepared by using precipitation method, in which two saturated aqueous solutions of sodium carbonate (Na_2CO_3) and calcium nitrate [$Ca(NO_3)_2$] were used. Solution A, in which Na_2CO_3 (5.3 g) was dissolved in deionized water (500 mL) and supplemented with 5 mL of 0.2 M sodium hydroxide (NaOH) and 5 mL of 0.18 M sodium nitrate ($NaNO_3$) aqueous solutions to provide alkaline pH suitable for calcium carbonate precipitation and to reduce the solubility of calcium nitrate by common ion effect respectively. Solution B, calcium nitrate (8.2 g) was dissolved in deionized water (500 mL), and then gently added to solution A with stirring at 300 rpm for 30 min. The mixture was poured in a separating funnel which allow to the nanoparticles of calcium carbonate to be settled down. Nanoparticles of calcium carbonate can be separated from the water by filter paper and then dried at room temperature (Mendham *et al*., 2000).

Qualitative test of proteolytic activity

There 100 mL of starch nitrate broth were prepared in two Erlenmeyer flasks (250 mL), but $CaCO_3$ was replaced by nano-sized of the same chemical and the same weight in the second flask. Starch nitrate broth is composed of soluble starch (20 g), KNO_3 (2 g), $MgSO_4$ (0.5 g), K_2HPO_4 (1 g), NaCl (20 g), $CaCO_3$ (3 g), trace salt solution (1 mL), and distilled water (1000 mL) at pH 7 ± 0.1. Trace salt solution is composed of $FeSO_4 \cdot 7H_2O$ (0.1 g), $MnCl_2 \cdot 4H_2O$ (0.1 g), $ZnSO_4 \cdot 7H_2O$ (0.1 g), and distilled water (100 mL). *S. spororaveus* was cultured by the same inoculum size (1 mL) in the two flasks, which incubated with shaking at 30 °C for 7 days and at 160 rpm. After incubation period has been finished, the bacterial suspension was filtered through a piece of cotton, and then the filtrate was centrifuged at 5,000 g for 10 min. The pellet was discarded, while the supernatant was pooled (cell free extract). On the other hand, starch casein agar plates were prepared. Starch casein agar is composed of soluble starch (10 g), casein (0.3 g), KNO_3 (2 g), $MgSO_4 \cdot 7H_2O$ (0.05 g), K_2HPO_4 (2 g), NaCl (2 g), $CaCO_3$ (0.02 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), agar (18 g), and distilled water (1000 mL) at pH 7.0 ± 0.1. Central well was made by sterile Cork Borer in each plate. This well was filled with the cell free extract of each flask (the first flask contained calcium carbonate, while the second one contained the calcium carbonate nanoparticles). Triplicates of plates for each flask were done. All plates were incubated at 30 °C for 24 h. After incubation period has been finished, all plates were visually examined. The positive result essentially accompanied with an appearance of clear zone around the central well and vice versa. The diameter of clear zone was measured by a ruler to determine the qualitative activity of protease enzyme (Larsen *et al*., 1998).

Quantitative test of proteolytic activity

Assay of proteolytic activity was carried out by addition of 250 µL of cell free extract to 500 µL of 1% (w/v) casein sodium salt (Sigma) and 50 mmol/L buffer (pH 7). This mixture was incubated for 20 min at 40 °C (Tsuchida *et al*., 1986). The reaction was stopped by addition of 1 mL of 20% (w/v) trichloroacetic acid to the mixture and left at room temperature for 15 min. The mixture was centrifuged at 10,000 *g* for 5 min to separate the non-reacted casein. The pellet was discarded, while the supernatant was mixed with 2.5 mL of 0.4 mol/L of

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Na_2CO_3 and 1 mL of 3-fold diluted Folin-Ciocalteu's phenol reagent, and then incubated at room temperature in the dark for 30 min and the absorbance of the blue color developed was measured at 660 nm against a reagent blank using a tyrosine standard (Lowry *et al*., 1951). One unit of enzyme activity was defined as the rate of releasing that gives rise to an increase of 0.1 units of absorbance in 1 h at 30 °C under the assay conditions (Tremacoldi and Carmona, 2005).

Assay of total protein content

The total protein content was determined according to Bradford (1976), where a standard curve of protein concentration was designed using bovine serum albumin (BSA).

Purification of protease

Ammonium sulphate precipitation

Protein content was precipitated in the supernatant by adding dry ammonium sulphate to 70% final concentration. The solution was centrifuged at 8,000 g for 20 min. All subsequent steps were carried out at 4 °C. The protein pellet was resuspended in gel permeation buffer (50 mmol/L NaH_2PO_4, 150 mmol/L NaCl, 1 mmol/L ethylene-diamine-tetra-acetic acid "EDTA"; pH 7) and dialyzed overnight against the same buffer.

Sephadex G-100 gel filtration chromatography

The dialysate was passed through a column chromatography (2.4 cm × 75 cm) compacted with a resin called Sephadex G-100. This resin was rinsed with gel permeation buffer to obtain an equilibrium. Several fractions were collected at a flow-rate 1 mL/min. The active fractions were determined and pooled.

Separation of protease

The purified protease in active fraction was separated by electrophoresis. On the other hand, standard proteins (markers) were used to determine the molecular weight of the purified protease (Laemmli, 1970).

Characterization of the purified protease

pH stability

Certain volume of purified protease was equally distributed in several tubes, each of which was supplemented with an equal volume of acetate buffer which has different pH value. All tubes were incubated at 37 °C for 30 min, and the activity of the enzyme was measured.

Thermal stability

Certain volume of purified protease was equally distributed in several tubes, each of which was supplemented with an equal volume of acetate buffer at an optimal pH value. Each tube was incubated at definite temperature (37, 50, 60, 70 and 80 °C) for 30 min and then the activity of the enzyme was measured.

Assay of antifungal activity

The antifungal activity of the purified protease was assayed by the disc diffusion method. The tested fungi were pre-cultured on PDA medium and adjusted at 10^5 CFU/mL, then added to PDA plates before solidification. Filter paper discs (7 mm in diameter) impregnated with the purified enzyme solution were placed on plates inoculated with the test organism. Three replicates were used. The activity was determined after 72 h of incubation at 28 °C. The diameters (mm) of the inhibition zones were measured.

RESULTS AND DISCUSSION

Qualitative test of proteolytic activity

The effect of calcium carbonate and calcium carbonate nanoparticles on the proteolytic activity of *S. spororaveus* was determined. The bacterial suspension was inoculated in two flasks contained starch nitrate broth, but the first one contained calcium carbonate and the second contained calcium carbonate nanoparticles. After 7 days of incubation has been finished, starch casein plates were inoculated by cell free extracts of the two media using agar well diffusion method. This test resulted in high proteolytic activity with nano-sized calcium carbonate more than that of an ordinary size of calcium carbonate (Figure 1). *Streptomyces* bacteria are well known as good producers for proteases, which are used in several types of industry including pharmaceuticals, detergents, and textiles (Simkhada *et al*., 2010; Manivasagan *et al*., 2013). Protease is an inducible enzyme, so the composition of the medium influences its production (Rifaat *et al*., 2007).

Quantitative test of proteolytic activity

The concentration of protease was assessed twice. The first time was carried out with presence of calcium carbonate, while the second time was carried out with presence of calcium carbonate nanoparticles. The assessment was carried out by measuring the absorbance at 660 nm using standard curve. *S. spororaveus* was found produced 150 µg/mL of protease with presence of calcium carbonate nanoparticles, but with presence of calcium carbonate this value was decreased to 65 µg/mL. This means that, calcium carbonate nanoparticles play an important role in raising the productivity of an extracellular protease (Figure 2).

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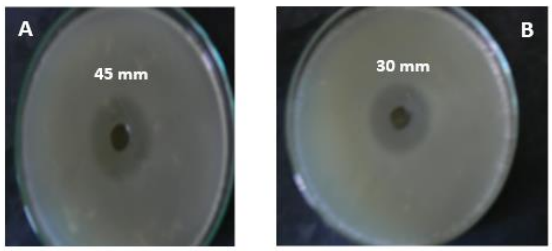

Figure 1: The effect of calcium carbonate nanoparticles (A) and calcium carbonate (B) on the proteolytic activity of *S. spororaveus*

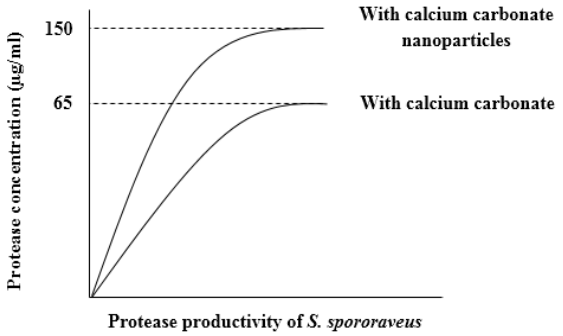

Figure 2: The effect of calcium carbonate nanoparticles and calcium carbonate on the protease productivity of *S. spororaveus*.

Purification of protease

After we have proved that the calcium carbonate nanoparticles considerably raised the productivity of protease more than calcium carbonate, it was essentially that the production medium (starch nitrate broth) contained calcium carbonate nanoparticles instead of calcium carbonate. The high production of protease was very essential for good isolation, purification, and characterization of protease.

An extracellular protease that produced by *S. spororaveus* was precipitated at 70% of saturated ammonium sulphate. At this concentration, the highest proteolytic and specific activities compared with the crude protease and other concentrations were obtained (Table 1). Abdulaziz *et al*. (2015) reported that, an extracellular alkaline protease was partially purified from the culture filtrate of *S. griseorubens* E44G, using the ammonium sulphate precipitation method. Maximum precipitation of protease content was reached at saturation of 70% of ammonium sulphate. Other studies stated that, proteases produced by *S. albidoflavus* were precipitated at 45% of saturated ammonium sulphate (Kang *et al*., 1995), and proteases produced by *S. alboniger* were precipitated at 40% of saturated ammonium sulphate (Lopes *et al*., 1999).

The precipitated active protein was dialyzed against the buffer, and passed through a Sephadex G-100 column chromatography. The fractions were collected and tested for protease activity, and the active fractions were pooled (Figure 3). Affinity chromatography is a common and suitable method for protease's purification process particularly Sephadex is a used resin (Rifaat *et al*., 2006; Prasad *et al*., 2014; Abdelwahed *et al*., 2014). Hatanaka *et al*. (2005) stated that, Sephadex G-75 was used for the purification of proteases from *S. albidoflavus*.

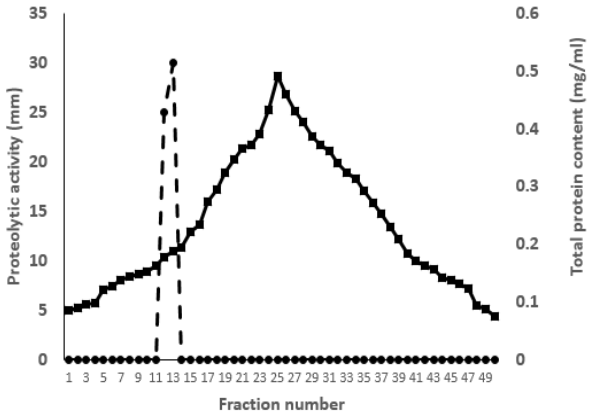

Figure 3: Purification of protease enzyme using Sephadex G-100.

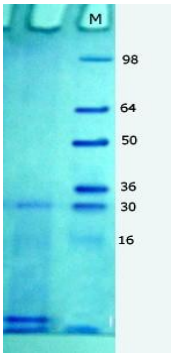

Figure 4: SDS-PAGE of the partially purified protease enzyme.

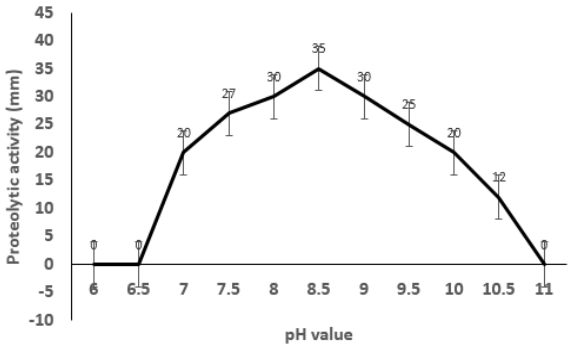

Figure 5: Effect of pH on the proteolytic activity.

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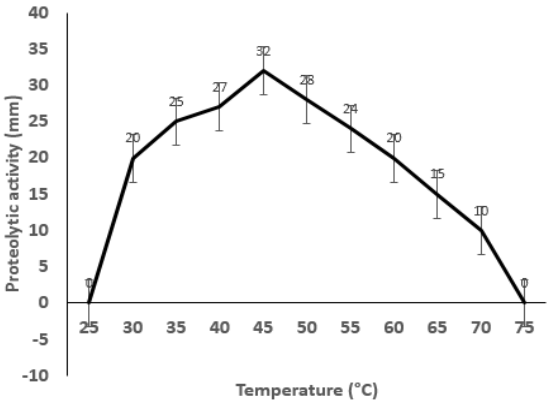

Figure 6: Effect of different temperature values on proteolytic activity.

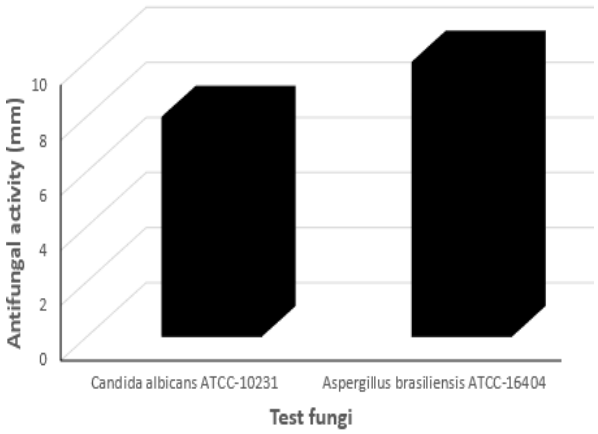

Figure 7: Determination of antifungal activity of the purified protease.

Separation of protease

The purified protease was separated as a single band at 30 kDa by using SDS-PAGE (Figure 4). Abdulaziz *et al*. (2015) reported that, the molecular weight of the protease enzyme (35 kDa) was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Characterization of the purified protease

pH stability

The effect of wide range of pH on protease activity was studied (6 – 11). This test revealed that, the highest protease activity was determined at pH 8.5 (Figure 5). However, more alkalinity led to sharp decreasing in the protease activity. Notably, we have no studied the effect of acidity on the proteolytic activity, because casein is quick sediment at acidic conditions. Alkaline proteases are commonly produced by *Streptomyces* bacteria at high levels (Abdelwahed *et al*., 2014).

Thermal stability

Usually alkaline protease can resist the raised temperature. So, the effect of different temperature values on protease activity was studied (25 – 75 °C). The proteolytic activity has been being observed at 30 °C and increased gradually until reached to culmination at 45 °C as an optimum temperature, and then decreased gradually until 70 °C and disappeared at 75 °C (Figure 6). Aftab *et al*. (2006) stated that, the highest proteolytic activity of *Actinomyces* sp. was obtained at ≥ 40 °C as an optimal temperature, so it requires a heated environment for optimum activity.

Determination of antifungal activity

The antifungal activity of the purified enzyme was determined by using disc diffusion method. The purified protease inhibited the growth of test fungi which named *C. albicans* ATCC-10231 and *A. brasiliensis* ATCC-16404 at 8 mm and 10 mm of inhibition zone respectively (Figure 7). Abdulaziz *et al*. (2015) reported that, the purified protease of *S. griseorubens* E44G inhibited the growth of *Rhizoctonia solani*.

Fraction		Activity (u)	Protein content (mg/ml)	Specific activity (u/mg)	Fold purification	Yield %
Crude protease		300	150	2	1	100
Saturated ammonium sulphate	10%	0	4.2	0	0	0
	20%	0	5.6	0	0	0
	30%	0	6.9	0	0	0
	40%	0	7.8	0	0	0
	50%	0	9.4	0	0	0
	60%	240	11.4	21.05	10.525	80
	70%	0	10.1	0	0	0
	80%	0	8.5	0	0	0
	90%	0	6.7	0	0	0
Sephadex G-100		260	10.9	23.8	16.05	86.6

Table 1: Precipitation of protease enzyme by saturated ammonium sulphate.

CONCLUSION

The activity of alkaline protease produced by *S. spororaveus* is significantly increased with calcium carbonate nanoparticles as one of constituents of starch nitrate broth. The highest activity of alkaline protease was obtained at pH 8.5 and 45 °C.

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