



PRODUCTION AND OPTIMIZATION OF THERMOSTABLE ALKALINE PROTEASE FROM *BACILLUS SP-APP-07* ISOLATED FROM LAUNDROMAT SOIL

Microbiology

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ABSTRACT

Laundromat soil consist many thermostable alkaline protease-producing microorganisms, hence screened for the isolation, identification, and production of thermostable alkaline protease. No such promising production medium defined for the production of alkaline protease, as a different level of enzyme production found with different medium constitutes. Therefore, we reviewed various cultural and media parameters to establish cost-efficient production medium for the strain APP-07.

In conclusion, we established the most suitable production medium for the newly isolated *Bacillus licheniformis*-APP-07 containing 1.0 % glucose, 0.5% yeast extract, 0.5% peptone, 5.0% NaCl, 0.02% MgSO₄, 0.1% K₂HPO₄, 0.02% CaCl₂, and 1.0% Na₂CO₃, 0.5 % defatted soy flour and 1.0% maltodextrin. The 774.969 U/ml of proteolytic activity was achieved after 33 h cultivation on a rotary shaker (100 rpm) at pH 10.5 and temperature 55°C. The enzyme may serve potential applications in detergents, tannery, food, pharmaceutical, diagnostics, protein biochemistry and many other industrial processes.

KEYWORDS

Alkaline protease, *Bacillus licheniformis*, Laundromat soil.

1. INTRODUCTION

Among all group of enzymes, proteases (EC 3.4.21) considered as a one of the most important class of enzyme and contributed more than 60-65% worlds share market. The global demand of the proteases increased exponentially and expected to reach up to 2.0 billion dollars in coming years.^{1,2} Thermo-stable alkaline proteases serve widespread applications in detergent, leather, peptide synthesis, silver recovery from X-ray films, pharmaceutical, diagnostic assays, dairy, food, beverages, and many other bioengineering industries.^{3,5}

The alkaline protease producing *Bacillus* sp. found its natural habitat in open-air laundromat (Dhobi Ghat) soil, which possesses attractive extracellular enzyme producing abilities with extreme pH and temperature tolerance.^{6,7} The enzymes from these strains belong to metallic type or serine centre proteases that sustain in harsh process parameters. The dual property and broad specificity of the enzyme had drawn the attention for the production of thermostable alkaline proteases.

Although higher pH and temperature stability of the enzyme is the primary goal, the cost obstacles kept open to search the new ways for the low-cost production of alkaline proteases.^{8,9} Several researchers reported that no such defined medium established for the production of the alkaline thermostable enzyme. Each strain has its own metabolite requirements and may vary according to genetic diversity and geographical conditions. About 40 % of the cost was spent on the production medium to achieve enhanced production.⁶ Therefore; optimization of low-cost production medium is an immediate requirement to obtain chief and affordable enzymes. Many researchers explored several approaches to achieve affordable, low-cost production mediums for maximum production of the enzyme by applying different variables like pH, temperature, and media compositions. Furthermore, several attempts exerted using agricultural and industrial by-products like corncob, green gram husk, wheat bran, rice bran, corn steep liquor, whey, sugarcane molasses, potato peels, soybean meal, feather meal, etc to reduce production cost.^{10,11}

In the current study, we aimed to isolate alkaline thermostable protease producing microbial strain from Laundromat soil and optimization of the low-cost production medium for the isolated strain to achieve improved cost-efficient enzyme production.

2. MATERIALS AND METHODS

2.1 Isolation and identification of selected strain

Soil samples from Laundromat (Dhobi Ghat) region were collected, serially diluted, and plated on 10% sterile milk agar plate-pH 10.5 (1.0% glucose, 0.5% yeast extract, 0.5% peptone, 0.1% K₂HPO₄, 0.2%

MgSO₄, 5.0% NaCl, 1.0% Na₂CO₃, 10.0% skimmed milk, and 1.5% agar). The skimmed milk, glucose, and Na₂CO₃ were sterilized separately, added aseptically to the media by adjusting pH 10.5 with Na₂CO₃. The plates were inoculated and incubated at 55°C for 24-48 h. After incubation, colonies showing maximum zone of clearance in their vicinity were picked up and subcultured on nutrient agar slants. The selected strain APP-007 was identified as *Bacillus licheniformis* based on their morphological, biochemical and 16S rRNA sequence analysis.

2.2 Production of alkaline protease

The same medium except skimmed milk and agar was used for the production of alkaline protease. 50 ml media was prepared in 250 ml Erlenmeyer flasks, 2% (v/v) inoculum inoculated and incubated on rotary shaker at 55°C for 72 h. The proteolytic activity was assayed after 24, 48, and 72 h growth.

2.3 Estimation of proteolytic activity

The proteolytic activity was measured according to Kembhavi et al.¹² method with some minor modifications. 1.0 % (w/v) casein prepared in 10 mM carbonate-bicarbonate buffer (pH 10.5) and incubated for 5 min at 55°C. Afterward, 1.0 ml of suitably diluted enzyme solution was added to the test solution, whereas blank test tube incubated for 15 min without addition of enzyme solution. The enzymatic reaction was stopped with 2.0 ml 0.4M trichloroacetic acid (TCA) solution. The reaction mixture allowed to stand for 25 min at room temperature (RT) by adding 1.0 ml enzyme solution in the blank test tube. Then the solution was centrifuged at 10,000g for 10 min and supernatant was assayed at 660 nm.¹³ The unknown concentration extrapolated against tyrosine standard curve (0-50 mg/ml). One unit of protease activity was defined as the amount of enzyme required to liberate 1 mg of tyrosine/ml/min under the standard experimental conditions

2.4 Measurement of total sugar and protein content

The total carbohydrate concentration in the sample was measured according to the phenol-sulphuric acid method described by Dubois et al.¹⁴ using glucose as a standard sugar, whereas protein concentration estimated according to Lowry et al.¹⁵ method using BSA as a standard protein.

2.5 Optimization of key determinants

The influence of aeration, agitation, incubation period, pH, and temperature were studied to optimize the cultural conditions for the microbial strain APP-07. The most suitable carbon, nitrogen, and various minerals were optimized by applying one-change-at-one-time (OCOT) approach system. All the optimization experiment performed by cultivating bacteria in 250 ml baffled flask with a working volume of 50 ml.

2.6 Effect of aeration and agitation

The influence of aeration and agitation on bacterial growth and enzyme production was studied by incubating 250 ml Erlenmeyer and baffled flasks at 50, 100, 150, 200, and 250 rpm on rotary shaker at 55°C up to 72 h. The proteolytic activity was assayed after 24, 48, and 72 h growth.

2.7 Effect of inoculum age and size on enzyme production

The influence of inoculums age and size were studied by inoculating 24 h and 48 h old inoculums at the concentrations of 2.5, 5.0, 7.5, 10.0, 12.5, and 15.0 % (v/v). The proteolytic activity was assayed after 24, 48, and 72 h growth.

2.8 Effect of various carbon and nitrogen sources on enzyme production

The effect of various sugars and agro-by product (fructose, sucrose, maltose, starch, lactose, mannitol, rice bran, maltodextrin, wheat bran, potato peels, and sugarcane molasses) on the protease production were studied by substituting glucose at the concentration of 1% (w/v). The effect of nitrogen was studied by replacing 0.5% yeast extract (w/v) with various nitrogen sources and agro-by products (beef extract, soybean meal, whey, urea, soy flour, defatted soy flour, potassium nitrate, ammonium chloride, and ammonium sulphate). The proteolytic activity was assayed after 24, 48, and 72 h growth.

2.9 Effect of temperature and pH on enzyme production

The optimum pH and temperature for maximum enzyme production were determined by assaying the optimized cultural media flask at different temperatures (40, 45, 50, 55, 60, 65, and 70°C) and pH (9.0, 9.5, 10.0, 10.5, 11.0, 11.5, and 12.0). The proteolytic activity was assayed after 24, 48, and 72 h growth.

2.10 Effect of incubation period on enzyme production

The optimum incubation period for maximum enzyme production was assessed using 100 ml optimized media prepared in 1000 ml baffled flask, inoculated with 10.0 % (v/v) inoculum and incubated at 55°C by maintaining pH 10.5 with sterile Na₂CO₃. The samples were removed after 3.0 h interval, the biomass and proteolytic activity were assayed up to 48 h.

2.11 Production of enzyme with optimized medium

Based on the media results most suitable media elements were selected in the final production medium, which includes (g/l): glucose (10), yeast extract (5), peptone (5), NaCl (50), MgSO₄ (0.2), K₂HPO₄ (1), CaCl₂ (0.2), Na₂CO₃ (10), defatted soy flour (5), maltodextrin (10). The glucose, Na₂CO₃, defatted soy flour, and maltodextrin were sterilized separately. The glucose added to the media pH adjusted to 10.5. After 12 h growth, defatted soy flour and maltodextrin were added aseptically to the medium and continued incubation up to 33 h on rotary shaker (100rpm) at 55°C. After incubation, media centrifuged at 10000g for 10 min and the clear supernatant was subjected for the determination of proteolytic activity.

2.12 Result Interpretation

All assays performed in triplicates and results were interpreted using GraphPad Prism 7 software.

3. RESULTS AND DISCUSSION

3.1 Isolation, identification, and production of enzyme

A total 18 morphologically distinct bacterial strains were screened for protease production, out of this isolate no APP-07 was found the potent microbial strain and identified as *Bacillus licheniformis* based on the morphological, biochemical, and 16s rRNA analysis. The microbial strain cultivated in a basal medium which results in 359.088 U/ml of the enzyme at 48 hr after that the productivity declined up to 72 h. The supernatant collected after 48 hr contained 16.389 U/mg of Specific activity among 21.91 mg/ml of total protein.

3.2 Optimization of key physicochemical determinants

The Erlenmeyer and baffled flasks were used to study the effect of aeration and agitation on bacterial growth and enzyme production. The alkaline protease production was highly affected by the aeration and agitation. The flasks cultivated with agitation showed maximum enzyme productivity compared to static flasks. The optimal cell growth and enzyme production was observed in baffled flasks, agitated on a rotary shaker at the speed 100-rpm (Fig. 1A and B). The result of inoculum age and size indicated that protease production was significantly improved when applied with 24 h old 10 % (v/v)

inoculums (Fig. 1C and D). Ibrahim et al. (2015) reported the microbial growth and enzyme productivity was greatly affected by the aeration, agitation, media pH, incubation temperature, age, and size of inoculums. The present study also observed with the noticeable influence of cultural parameters during alkaline protease production.

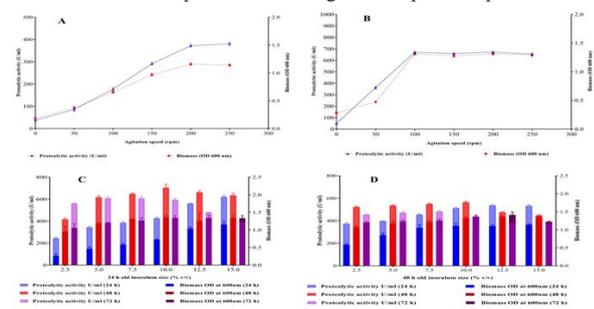


Fig. 1. Effect of aeration, agitation, inoculum age, and size on bacterial growth and alkaline protease production. (A) Influence of agitation on growth pattern and enzyme production in Erlenmeyer flask (B) baffled flask. (C) Demonstrate 24h old inoculum influence on enzyme production, whereas; (D) depict 48h old inoculum influence. The values represented are the mean values (n = 3) and standard deviation demonstrated by error bars.

3.3 Optimization of appropriate carbon and nitrogen sources by OCOT approach

The media optimization performed using OCOT approach for the suitable media ingredient. *Bacillus licheniformis* showed maximum enzyme productivity in presence of glucose (714.566U/ml) and maltodextrin (608.357 U/ml) as a carbon source, while yeast extract (753.066 U/ml) and defatted soy flour (694.976 U/ml) as the best nitrogen source (Fig. 2).

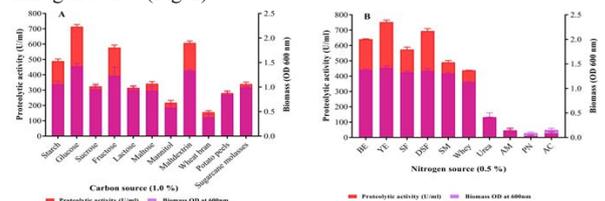


Fig. 2. Effect of various carbon and nitrogen sources on bacterial growth and alkaline protease production. (A) Indicates the effect of carbon source, whereas; (B) Nitrogen source (BE=beef extract, YE= yeast extract, SF= soy flour, DSF= defatted soy flour, SM= soybean meal, PN= potassium nitrate, AC= ammonium chloride, and AS= ammonium sulfate). The values represented are the mean values (n = 3) and standard deviation demonstrated by error bars.

3.4 Effect of incubation temperature and pH

The influence of temperature and pH on the productivity of enzyme was studied using optimized media cultivated at different pH and temperatures. The maximum enzyme production 752.501U/ml and 753.186 U/ml observed at pH 10.5 and 55°C respectively. The effect of various temperatures and pH on enzyme production was demonstrated in Fig. 3.

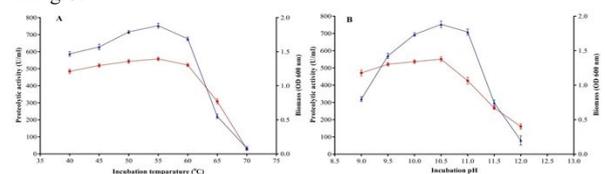


Fig.3. Influence of incubation temperature and pH on bacterial growth and alkaline protease production. (A) Demonstrate the effect of incubation temperature, whereas; (B) Effect of incubation pH. The values represented are the mean values (n = 3) and standard deviation demonstrated by error bars.

Many researchers recognized that alkaline protease productivity is greatly influenced by physicochemical and production media parameters which include aeration, agitation, pH, temperature, incubation time, concentration of substrate, ions, sugars, and salts which played an important role in the optimal production of

enzymes.^{9,10,16} Furthermore, Kshetri et al. (2016) represented the statistical approach one-variable-at-a-time to establish low-cost optimal medium for alkaliphilic *Bacillus* sp. KW2 using various agricultural by-products.

3.4 Effect of incubation period on enzyme production

The influence of incubation period on enzyme production was assessed using 100 ml production medium. The optimum incubation period observed at 33 h with highest enzyme activity (774.969 U/ml) afterward a very minute change observed in enzyme production. The secretion of alkaline protease was coherent with growth pattern and exponentially enhanced with an increment of growth (12-18 h). The highest secretion observed at the initiation of stationary phase. The effect of incubation period on bacterial growth kinetics and enzyme production was demonstrated in Fig. 4.

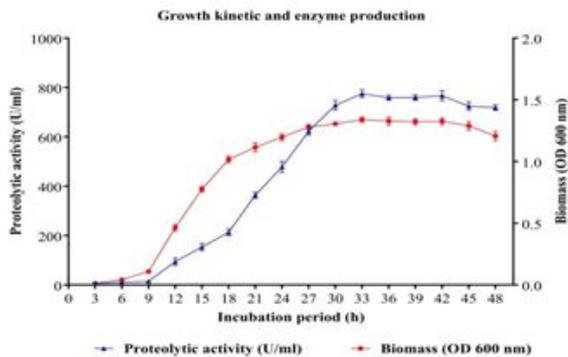


Fig. 4. Bacterial growth kinetics and alkaline protease production. The values represented are the mean values (n = 3) and standard deviation demonstrated by error bars.

CONCLUSION

In the conclusion, *Bacillus licheniformis*-APP-07 isolated from Laundromat soil exhibited potent thermostable alkaline protease producing ability that shows 774.969 U/ml of enzyme activity in the optimized production medium. The intermediate feeding of defatted soy flour and maltodextrin enhances the productivity of enzyme. The crude enzyme showed the wide range of pH and temperature tolerance with optimal activity at 55°C and pH-10.5. The enzyme may serve potential applications in laundry detergents, tannery, food, pharmaceutical, diagnostics, protein biochemistry, and many other industrial processes. Further, a complete large-scale process optimization, purification, and characterization work is needed to achieve affordable production of thermostable alkaline protease.

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