

Effect of Physical and Chemical Factors in Production of Alkaline Protease Enzyme by *Bacillus* Strains

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Abstract. Proteases is family of enzymes and it has crucial role due to their physiological roles and very valuable commercial applications. Alkaline protease are produced by *Bacillus* species are particular importance because of their thermal stability and stability at different pH values. This study aimed to investigate the effect of physical and chemical factors in production of alkaline protease enzyme fermentation by members of the genus *Bacillus*. In this study, alkaline protease enzyme production were evaluated in submerged fermentation by *Bacillus* strains which were isolated from alkaline soils of Guilan province. Factors incubation were optimized such as time, pH, amount of inoculation and ammonium sulfate in alkaline protease enzyme production whit using response surface methodology (RSM) in culture. The maximum enzymatic activity was observed in incubation time of 36 hours, pH=9, inoculation amount of 15% (V) and ammonium sulfate 1.5% (W/V). Factors had significant effect on the production of alkaline protease enzyme such as pH and ammonium sulfate.

Introduction

Proteases are a family of enzymes and they have very an important position because of their physiological and commercial applications. Proteases are physiologically required for living organisms and there are in a wide range of natural sources such as plants, animals and microorganisms. Proteases plays two role of proteolytic and synthetic and their functions have been detected form cell surface to organs and organisms [1, 2]. Alkaline proteases are known as ubiquitous in nature. Alkaline proteases are isolated from several sources by surface plating and subsequent screening [3]. Microbial proteases have known as multifunctional enzymes and they can catalyze hydrolysis of proteins to amino acids. Alkaline proteases are the most normally used industrial enzyme among different types of proteases because of high activity and stability [4, 5]. Proteases have numerous commercial applications and among the variety of enzymes, microbial proteases have been used in detergents, pharmaceuticals, industrial enzymes, producing silk and 60% of total sales of industrial enzymes assigned to this enzyme [1]. Microbial proteases have advantages which are better than a variety of animal and plant resources including: a variety of biochemical, genetic manipulation capabilities, rapid growth, and limited space required for growth [2, 6-10]. Proteases are categorized according to their source and also, according to the Enzyme

Commission organization, proteases are class three (hydrolases), and sub-group four (which hydrolyse peptide bonds) [3]. Proteases are divided in 3 types of acidic, neutral and alkaline based on the optimal pH range. Alkalophilic microorganisms are widely distributed in nature and almost can be found in any environment. However, some specific environments have alkaline substances such as soil, lakes and deserts of alkali. *Bacillus* have an important role in industry among microorganisms and they are a rich source of alkaline proteases [11-13]. The reaction of protease is affected by some factors, for example pH, ionic strength, Tm and mechanical handling. Novel enzymes constantly are demanded with special properties in the industrial process [3]. The production of this enzyme are performed with both submerged and solid state fermentation culture. Alkaline protease are produced by submerged fermentation and solid state fermentation processes are used less [8, 14, 15]. The pH of culture disturbs all enzymatic processes and transport of numerous constituents across the cell membrane [3]. pH should be kept more than 7.5 for increasing the efficiency of protease production during fermentation [8, 14, 15]. This research aimed to optimize the parameters of time, pH, inoculum and ammonium sulfate for producing the alkaline protease by submerged fermentation.

Methods and Materials

Sample collection and isolation of microorganisms

15 soil samples were collected from southern areas of Manjil, Lushan and Roodbar (Gilan Province) and transferred in sterile plastic bags to lab. Then, 1 gr of each samples was prepared in 9 ml of distilled water and was heated for 20 minutes at a temperature 80 °C. After that, 0.1 ml of them each was transferred to nutrient agar medium and then, were cultured and incubated for 24 hours at 37 °C. Finally, the colonies were identified by biochemical test [16].

Screening of strains producing enzyme

All *Bacillus* strains were cultured in the medium skim milk powder (20 g per liter) and agar (20 g per liter) and was kept at 37 °C for 24 hours. Producing of a clear halo around the colony represents the protease enzyme production [17].

Identification of strains

Identification of strains was confirmed by microscopic examination, Gram staining, biochemical tests, Gelatinase experiment, hydrolyzed casein, catalase, hydrolysis of lecithin, urease, decomposition citrate, reduction of nitrate, hydrolyzed starch and sugar fermentation.

Preparation of cultures and Statistics optimization of protease production by RSM

A source of bacterial inoculation medium was prepared containing glucose (10 g per liter), peptone (5 g L), yeast extract (5 g per liter), casein (2 grams per liter), Di-potassium hydrogen phosphate (1 g per liter), Epsom 7 water (2.0 g per liter) per 50 ml were taken and the pH value of 10 was set using a pH meter [12]. The efficiency of enzyme production was evaluated by this method. Central composite design includes four independent variables; incubation time (C1), pH (C2), inoculated (C3), the amount of ammonium sulfate (C4) for fermentation in submerged fermentation. 30 tests were designed for submerged fermentation for each factor to zero using these four factors and five levels coded [18].

Determine the accuracy of the response surface model

Submerged fermentation method was tested experimentally after determining the amount of production (based on software prediction) and the results were compared with predicted results [18].

Optimization of submerged fermentation production by RSM

The culture medium was prepared containing 50 ml of distilled water and glucose (10 g per liter), di-potassium hydrogen phosphate (1 g per liter), magnesium (0.2 grams per liter), casein (1%)

and ammonium sulfate were added in each of 30 experiments and pH was measured by pH meter. Then, flasks were sterile for 20 minutes in an autoclave at a temperature 121 °C. The inoculation rate was determined according to table 1 and was added to media. Enzyme production was examined after passing time [19].

The effect of environmental parameters on enzyme production in submerged fermentation

Culture media were studied in 12, 24, 36, 48, 60 h, pH (7, 8, 9, 10, 11), the inoculation with volume % (5, 10, 15, 20, 25) and ammonium sulfate weights % (0.5, 1, 1.5, 2, 2.5).

Enzyme extraction

For this purpose, the liquid medium was filtered from filter and poured in test tubes and then, was centrifuged for 20 min at 6000 rpm. The supernatant was used to assess the enzymatic activity as a solution containing the enzyme.

Assessment Activity of enzyme

The experiment was performed with casein substrate. 200 µl of casein substrate solution (containing 20 grams of casein in 1000 ml Tris) were added to 2 ml of enzyme solution and then, incubated for 20 min at 35 °C. 2/5 ml TCA (5% TCA, 9% and 9% acetic acid) was added in the solution and held for 5 minutes at room temperature to stop the enzymatic reaction. Then, the tubes were centrifuged for 20 min at 6000 rpm and then, the optical density of the samples was measured at 280 nm by a spectrophotometer. A control tube was prepared containing 0/2 ml distilled water, 2 ml substrate casein and 2/5 ml TCA solution. The effect of pH was evaluated from pH 7–11, using sodium phosphate buffer (pH 7.0–8.0) and glycine NaOH (pH 9.0–11.0) buffers. A unit of enzyme activity was defined as the amount of enzyme that can produce one mg tyrosine per minute. The amount of tyrosine results were determined with using standard curve [7].

Statistical analysis

30 tests were designed by RSM design and analyzed by using the SPSS (ANOVA) software. The level of statistical were considered significant difference ($P < 0.05$).

Results

The results of biochemical tests confirmed that this bacteria belonging to *Bacillus* genus (Table 1). Analysis of variance (ANOVA) showed that pH and ammonium sulfate have a significant effect on enzyme production ($P < 0.05$). The maximum amount of enzymes production was in pH =9, inoculation rate of 15% (volume by volume), ammonium sulfate 1/5 percent (weight by volume) and 36 hours.

Table 1. Biochemical characteristics of the isolated *Bacillus*

Biochemical test	Result
Hydrolysis of casein	+
Hydrolysis of starch	+
Hydrolysis of gelatin	+
Hydrolysis of lecithin	+
Catalase	+
nitrate	+
Urease	-
Analysis of citrate	-
movement	+
Arabinose	-
Maltose	+
Mannitol	-
Xylose	+

Three-dimensional graphs of enzyme production in submerged fermentation by RSM

The effect of pH and Ammonium Sulfate was shown in Fig. 1 and Fig. 2 on the production of alkaline protease. Fig. 1 and Fig. 2 show an alkaline protease production. Enzymatic activity was increased in the concentration of ammonium sulfate 1% with increasing in pH (range 7 to 9) and then it gradually reduced when arriving at the point of maximum pH= 9 (the incubation time (36 hours) and inoculation rate (15% (volume by volume))).

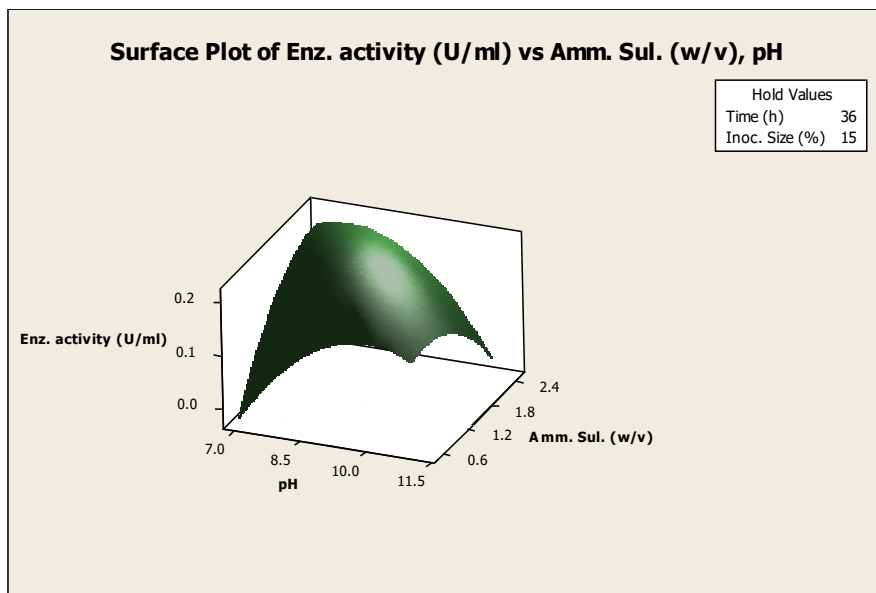


Figure 1. The effect of enzyme activity in the production of alkaline protease by pH and Ammonium Sulfate

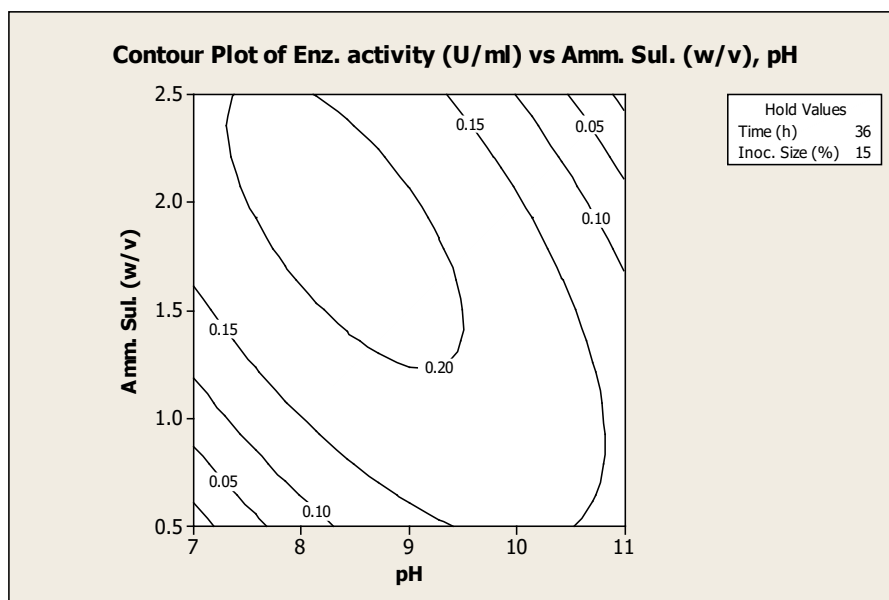


Figure 2. The contour plot of enzyme activity in the production of alkaline protease by pH and Ammonium Sulfate

Discussion

Alkaline protease is an important group of enzymes and commercial physiologically as additives in detergents. Biotechnology applications of alkaline protease enzyme have been increased to business goals and also, optimize production medium was suggested for increasing the productivity. Extracellular proteases have numerous applications in various industrial sectors. Although, there are many microbial resources for enzyme production, but few of these resources are

industrial value [20, 21]. RSM is an experimental design which has advantages over traditional methods. Traditional methods optimization take time and cross-reaction between variables physical and chemical processes cannot be checked. However, RSM can described the incubation time, pH and the percentage of ammonium sulfate as individual and interaction on alkaline protease production [20]. The best way to determine the effect of independent variables on the dependent variable draws graphs response surface model, which is done by two different variables within the scope of testing and fixing two variables at zero level [20]. According to the obtained results, optimal levels of closed culture was predicted by Minitab 16 software for maximum efficiency of alkaline protease. It predicted time= 37.6970, pH =8/37374, inoculated 19.3434% and Sulfate Ammonium 1. 77273 percent for maximum production (0.329U / ml). In this research, enzyme activity (0.318ml U) was observed that this represents a model of conformity between the predicted results and experimental results. A slight difference between the predicted value and the observed value can be caused by changes in testing conditions. pH is an important factor in production of alkaline protease enzyme. Changes in enzyme production depends on the pH value changes. PH conditions vary from one species to another. This change is also seen in different species which isolated from a habitat [12, 22].

The results of this research showed that gradually reduce the production of the enzyme were found with increasing the pH to the optimum value (pH =9). The increase and decrease in enzyme activity could be due to the effect of pH on the growth of microorganisms, changes in ionic charge of enzymes and transfer material from membrane [23]. In this study, the maximum production of enzymes was observed in pH = 9 which is similar to other researches. Ammonium sulfate was another significant factor. Microorganisms metabolized both organic and inorganic forms of nitrogen to produce amino acids for producing nucleic acids, proteins and cell wall components. Alkaline protease contains nitrogen (15.6 percent) and its production depends on access to sources of carbon and nitrogen in the medium. Although, complex nitrogen source was used for the production of alkaline protease, it requires a suitable nitrogen source depending on the type of organism. Ammonium sulfate is considered as a mineral nitrogen source which is an appropriate source in the production of enzymes [24]. In this study, ammonium sulfate (0.5, 1, 1.5, 2 and 2.5) % (w v) were studied. Maximum enzyme activity related to experiment with ammonium sulfate 1.5% (W/V). Enzyme activity was 0.160 U/ml in similar conditions with 0.5% of ammonium sulfate and reduction of enzyme activity (0/130 U / ml) were obtained with increasing the amounts of ammonium sulfate to 2.5% (W/V). Imtiaz and colleagues in similar studies showed that optimal concentration of nitrogen source to produce varying amounts of alkaline protease enzyme was 1.5 % of ammonium sulfate [24].

Results also showed that enzyme production process was increased by increasing the concentration of inorganic nitrogen to 1.5% but the production of enzyme was reduced when concentrations was higher than 1.5%. The amount of nitrogen is an essential factor for the enzyme production in the culture medium. Therefore, nitrogen source is critical factor with an appropriate concentration for producing enzyme [24]. Alkaline Protease was produced by *Bacillus* strains with immersed fermentation method (36 h, pH= 9, inoculation rate of 15% (volume by volume) and ammonium sulfate 1.5 %) and also, pH and ammonium sulfate had significant effect in production of alkaline protease.

Conclusion

It can be concluded that time, pH, amount of inoculation and ammonium sulfate had important effects on the production of alkaline protease enzyme which The maximum enzymatic activity was detected in incubation time of 36 hours, pH=9, inoculation amount of 15% (V) and ammonium sulfate 1.5% (W/V).

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Conflict of Interest

The authors declare that there is no conflict of interest.

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