Influence of process parameters on the production of detergent compatible alkaline protease by a newly isolated Bacillus sp. Y.

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Abstract: Detergent compatible alkaline protease production by a newly isolated Bacillus sp. Y from laundry soil was studied under shake flask conditions in growth medium comprised (g/L): peptone, 1; NaCl, 5; skim milk, 100; Na₂CO₃, 4. Different environmental process parameters such as fermentation period, initial pH, incubation temperature, and nutritional parameters such as supplementation of different starch sources and nitrogen sources were standardized for the maximum yield of alkaline protease. Rice flour showed an almost 2.2-fold increase in protease activity followed by wheat flour, which showed a 1.85-fold increase over the basal media. However, supplementation of potato starch, corn starch, and ragi exerted an inhibitory effect on alkaline protease production. Protease production by Bacillus sp. Y was suppressed up to 80% in the presence of most of the organic and inorganic nitrogen sources tested in comparison to rice flour:peptone supplementation. Hence, based on the optimization studies, we achieved a yield of 88 PU/mL (2.2-fold increase) with the Bacillus sp. Y when cultivated for 72 h at pH 7.5, 37 °C in a medium containing: rice flour (1%, w/v), NaCl (0.5%, w/v), and skim milk (10%, v/v) with Na₂CO₃ (0.4%, w/v).

Key words: Alkaline protease, Bacillus sp. Y, optimization, cheap substrates

Introduction

Proteases are essential constituents of all forms of life on earth including prokaryotes, fungi, plants, and animals. They are highly exploited enzymes in food, leather, detergent, pharmaceutical, diagnostics, waste management, and silver recovery. The protease enzyme constitutes two-thirds of total enzymes used in various industries and this dominance in the industrial market was expected to increase by the year 2005 (1). Of all proteases, alkaline proteases produced by Bacillus species are of great importance in the detergent industry due to their high thermo stability and pH stability. For production of enzyme for industrial use, isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process (2).

Generally, proteases produced from microorganisms are constitutive or partially inducible in nature, and their production is mostly under the influence of culture conditions (3). Bacillus species are major producers of proteolytic enzymes, which are produced during post-exponential and stationary phases of growth. Extracellular protease production from Bacillus species is significantly influenced by
medium composition and some physical factors, such as fermentation period, aeration, inoculum density, pH, and incubation temperature of growth medium (4-6). Cost of the enzyme is one of the main factors determining the economy of the process. Reducing the cost of enzyme production by optimization of the fermentation medium and process parameters is the major goal of basic research for industrial applications (7). In fact, the optimization of different process parameters and the quantity of medium ingredients are basic tools for making enzyme production economically feasible.

This study focused on formulation of a medium that substantially enhances synthesis and secretion of detergent compatible alkaline protease in cultures of a newly isolated Bacillus sp. Y (8). The series of experiments conducted to identify culture conditions that lead to improved protease production also enable investigation of the regulatory effects of important culture parameters including effect of different organic and inorganic nitrogen sources, different carbon sources, as well as protease production in this bacterium, which further facilitates economic design of the large scale fermentation operation system.

Materials and methods

Microorganism

The microorganism used in this study was isolated in a previous study from local laundry wash areas in and around Bangalore, India, screened using a skim milk agar plate in alkaline broth. It was identified as Bacillus sp. according to morphological and biochemical tests (8). Analysis of the partial nucleotide sequence of the 16S rDNA (~1.5kb) identified the isolate to be most similar to Bacillus sp. Y (Gen Bank entry: ABO 55095), with a close homology to B. cohnii YN-2000 (Gen Bank entry: ABO23412).

Culture maintenance

Stock cultures of the isolate were stored in 30% (v/v) glycerol at −70 °C. Prior to each experiment, the bacterium was subcultured from the frozen stocks onto a solid alkaline medium as basal medium (pH 10.5) containing (g/L): peptone, 1; NaCl, 5; skim milk, 100; Na₂CO₃, 4.

Seed culture medium

For enzyme production, bacterial cells from a 24 h aged culture were inoculated into 250 mL Erlenmeyer flasks containing 50 mL of sterile inoculation medium. The composition of the inoculum medium was the same as described for culture maintenance. The cultures were grown at 37 °C on a stirrer at 150 rpm for 16-18 h. After reaching an optical density of about 1.0 at 600 nm, 2% (v/v) of the culture was used to inoculate production flasks.

Time course study for optimal alkaline protease production

Optimal time for protease production at pH 7.0 and 37 °C was studied by harvesting the production media at different days (0-7 days) and determining the protease activity.

Effect of media pH on protease production

To determine the effect of growth media pH on protease production the basal media with different pH (7-12) was inoculated and incubated at 37 °C for 48 h. Media pH was adjusted with 1N NaOH.

Effect of different starch and nitrogen sources on alkaline protease activity

Different kinds of starch sources [corn starch, potato starch, wheat bran, wheat, ragi (millet), rice flour] and nitrogen sources (NH₄Cl, KNO₃, NaNO₃, NH₄SO₄, NH₄PO₄, urea, beef extract, tryptone) were supplemented individually to the cultivation medium. All these sources were added to the production medium at a final concentration of 1% (w/v) for starch and 0.1% (w/v) nitrogen, respectively. The initial pH of the medium was set at 7 and with no control of pH during the shaker-flask cultivation. The protease yield was determined after 72 h of incubation at 37 °C under shaking (150 rpm).

Protease assay

Enzyme was assayed by using casein as a substrate as described by Kumar et al. (9) using sodium carbonate buffer (100 mM, pH 10). Tyrosin standard solution in the range of 0-1000 mg/L was prepared in triplicate to obtain a standard curve. One alkaline protease unit (U) was defined as the enzyme amount that could produce 1 μg of tyrosine in 1 min under the defined assay conditions.
Chemicals used and statistical analysis

The chemicals used for all of the experiments were of analytical grade. All the experiments were carried out independently (in triplicate) in 250-mL Erlenmeyer flasks. The data represented here are in the form of mean ± SD. All the values were subjected to one way analysis of variance (ANOVA) and significance is presented as Duncan’s multiple range test results in the form of probability (P ≤ 0.05) values, which were obtained using MSTAT software.

Results and discussion

The most significant outcome of the present study was optimization of the fermentation process parameters and kinetic studies for alkaline protease production using cheaper and easily available substrates by Bacillus sp. Y. It is well known that the proper optimization of process parameters plays an important role in improving enzyme yield, making enzyme production cost effective and economically feasible.

Time course study for optimal alkaline protease production

Bacillus sp. Y showed a gradual increase in protease titer from day 1 at pH 7.0 and 37 °C with the maximum at day 3 (40 PU/mL) followed by which there was a gradual decrease up to day 6 (Figure 1), which may have been due to insufficient availability of some nutrients in the growth medium. The cost of enzyme production depends mostly upon the operating cost; therefore, a shorter fermentation period would increase profitability on an industrial scale, compared to the added yield obtained with long incubation periods.

Maximum enzyme production was observed after 96 h with alkalophilic Bacillus sp. isolated from natural habitats (5). A broad incubation period ranging from 48 to 70 h for maximum protease enzyme yield by Bacillus strains has been previously reported (6,10). The main advantage of enzyme production by Bacillus sp. Y is a shorter incubation period, which will decrease operational cost, as well as autolysis of the enzyme created by protease itself during the fermentation process.

Effect of media pH on protease production

From an industrial prospective, the protease must exhibit significant activity and stability at high pH(s) and temperature(s). The effect of initial pH was investigated in order to determine the most suitable pH for the growth of Bacillus sp. Y and alkaline protease production (Figure 2). Bacillus sp. Y produced extracellular protease when cultured in a basal medium with different pH (7-13). The highest alkaline protease yield (60 PU/mL) was recorded at an initial growth medium pH of 7.5. These findings indicate that Bacillus sp. Y is sensitive to change in pH because change in growth medium pH affects growth and protease production. It has been noted that the most important characteristic of microorganisms is their strong dependence on extracellular pH for cell growth and enzyme production (11); therefore, it is concluded from the present experimental results that adjustment of initial growth medium pH is necessary for maximum alkaline protease yield by Bacillus sp. Y. Maximum alkaline protease production has been reported in different pH ranges (i.e. 7-11) for different Bacillus spp. (5,12). pH requirements vary from species to species and even in different strains of the same species isolated from different habitats.
Effect of growth temperature on protease production

Temperature is one of the most critical parameters to be controlled in any bioprocess (13). The effect of temperature on alkaline protease production revealed that maximum yield was obtained at 37 °C (70 PU/mL) (Figure 3). A decrease in enzyme yield was observed with further increases in temperature; hence, production of alkaline protease by Bacillus sp. Y was determined to be growth-related, which is a common phenomenon in many fermentation processes. The temperature-associated decreases in yield may have been due to the fact that denaturation or degradation of the proteolytic enzyme by autolysis in response to elevated temperatures caused alkaline protease activity to decrease. It was also reported that most alkaline Bacillus strains are mesophile types, with optimal temperatures ranging from 30 to 37 °C (4,14).

Effect of different carbon sources on alkaline protease activity

The use of cheap sources of carbon and nitrogen like wheat bran, rice bran, casein, and soy meals is important as these can significantly reduce the cost of production of protease. Therefore, the effects of various carbohydrates and organic nitrogen sources were evaluated at optimum pH, temperature, and incubation time with respect to enzyme yield. It was reported earlier that addition of starch as carbon source to the culture medium induced protease synthesis and the production was dependent on starch type (15). When the effect of different starches as carbon sources on protease production was assessed, wheat flour, wheat bran, and rice flour showed an increase in the protease yields. Rice flour showed an almost 2.2-fold increase followed by wheat flour, which showed a 1.85-fold increase over the basal media. However, supplementation of potato starch, corn starch, and pearl millet flour exerted an inhibitory effect on alkaline protease production (Figure 4). This negative influence on enzyme synthesis may be due to the presence of protease inhibitors in these sources (16). The addition of other easily metabolizable sugar like glucose reduced enzyme production drastically although growth was observed. This negative effect of glucose on protease production is attributed to catabolite repression (9,17-19) and was similar to that observed for Bacillus horikoshii as reported previously (20). In some Bacillus strains such as Bacillus sp. AR 009 (21) and B. licheniformis ATCC 21415 (22), however, enhanced protease yields were reported on supplementation of glucose.
Effect of different nitrogen sources on alkaline protease activity

The effect of addition of various organic and inorganic nitrogen sources (0.1%) replacing peptone on protease activity is shown in Figure 5. Among the various organic and inorganic nitrogen sources, all the nitrogen sources supported protease production. Protease production by Bacillus sp. Y was suppressed up to 80% in presence of most of the organic and inorganic nitrogen sources tested in comparison to peptone supplementation. It was earlier reported that the addition of casein substantially improved the protease production in Bacillus licheniformis MIR29 and Bacillus sp. (4,23). Protease production was increased approx. 30% by the addition of 1% (w/v) casein in Bacillus horikoshii isolated from the hemolymph of a unique Korean polychaeta, Periserrula leucophryna (20). In contrast, Joo et al. (13) reported the addition of casein and gelatin showed no or little effect on the protease production in Bacillus clausii I-52. Our result is in accordance with this report. The cost of the growth medium is another significant parameter for making the production process industrially viable. Approximately 30%-40% of the production cost of industrial enzymes is estimated to be accounted by the cost of the growth medium (21). With respect to the nitrogen sources, soybean meal (Glycine max) is one of the potentially useful cost-effective medium substrate because of its easy availability and low cost as it is produced as a by-product during oil extraction (22). Bacillus species have been successfully used in degradation of proteinaceous waste into useful biomass by many investigators (24-26).

Conclusions

Since our isolate shows appreciable protease production in wheat bran and soybean meal media, in addition to utilizing rice flour and ragi flour, this is important from the view point of cost effective production, as soybean meal and wheat bran is one of the cheap and readily available medium ingredients. Based on the optimization studies, we achieved a yield of 88 PU/mL (2.2-fold increase) from 40 PU/mL in basal media pre-optimized with the Bacillus sp. Y when cultivated for 72 h at pH 7.5, 37 °C in a medium containing (g/L): rice flour (1%, w/v), NaCl (0.5%, w/v), peptone (0.1%, w/v), and skim milk (10%, v/v).
with Na₂CO₃ (0.4%, w/v). In this study, we further demonstrate that *Bacillus* spp. are useful in deproteinisation of agro wastes like wheat bran, rice bran, and soya meal, and can be used as production medium for alkaline protease, which can find application in the detergent industry.

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