

Optimization of Process Parameters for Alkaline Protease Production by *Bacillus licheniformis* N-2 and Kinetics Studies in Batch Fermentation

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Abstract: Production of alkaline protease from *B. licheniformis* N-2 was studied in shake flask experiments in growth medium comprised of (g/l): glucose, 10; soybean meal, 10; K₂HPO₄, 5; MgSO₄.7H₂O, 0.5; NaCl, 0.5; CaCl₂.2H₂O, 0.5. Different process parameters, such as fermentation period, initial pH, incubation temperature, agitation speed, and size and age of inoculum, were optimized for the maximum yield of the alkaline protease. Maximum enzyme production (991.04 PU/ml) was obtained after 24 h of incubation at 37 °C in growth medium of pH 10. The optimum inoculum size and age were determined as 2.0% (v/v) and 24 h, respectively. Kinetics of growth and protease production by this microorganism were also analyzed at all optimum conditions. Biomass ($Y_{x/s}$) and product yield ($Y_{p/s}$) coefficients were determined as 0.375 g of cells/g of glucose and 99.3 PU/g of glucose, with a growth rate of (0.215 h⁻¹) after 24 h of incubation. Thereafter, a continuous decrease in biomass and product yield was observed. The results indicate that enzyme production by *B. licheniformis* N-2 was entirely growth-associated and that a major portion of the enzyme was secreted in the post exponential phase.

Key Words: Process parameters, growth kinetics, alkaline protease, *Bacillus licheniformis* N-2

Bacillus licheniformis N-2 Aracılığı ile Alkalın Proteaz Üretiminin İşlem Parametrelerinin Optimizasyonu ve Batch Fermentasyonla Kinetik Çalışmalar

Özet: *B. licheniformis* N-2 bakterisinden alkalın proteaz üretimi için çalkalamalı erlenlerde, 10 glukoz, 10 soya fasulyesi unu, 5 K₂HPO₄, 0,5 MgSO₄, 0,5 NaCl ve 0,5 CaCl₂.2H₂O (g/l) ihtiva eden büyüme besiyeri ortamında çoğaltılmıştır. Maksimum alkalın proteaz ürünü elde etmek için fermentasyon zamanı, başlangıç pH, inkubasyon sıcaklığı, çalkalama hızı, inokulumun büyüklüğü ve yaşı gibi farklı işlem parametreleri denenmiştir. Maksimum enzim üretimi (991,04 PU/ml) pH 10 olan besiyeri ortamında 37 °C de 24 saat inkubasyon sonrası elde edilmiştir. Optimum inokulasyon oranı ve inokulumun yaşı % 2 (v/v) ve 24 saat olarak belirlenmiştir. Bütün optimum şartlarda bu organizma tarafından üretilen proteaz ve büyüme kinetiği incelenmiştir. 24 saat inkubasyon sonrası biyokütle ($Y_{x/s}$) ve ürün ($Y_{p/s}$) katsayısı. 375 g hücre/g glukoz ve 99,3 PU/g glukoz, büyüme oranı (0,215 h⁻¹) olarak hesaplanmıştır. Daha sonra biyokütle ve ürün miktarında devam eden bir azalma gözlemlenmiştir. Sonuçlara göre *B. licheniformis* N-2 suşu ile enzim üretimi tamamen büyüme ile ilgili ve enzimin en önemli miktarı eksponensiyel faz sonrası salgılanır.

Anahtar Sözcükler: İşlem parametreleri, büyüme kinetiği, alkalın proteaz, *Bacillus licheniformis* N-2

Introduction

Enzymes are extremely efficient and environmentally friendly protein catalysts synthesized by living systems. They have significant advantages over chemical catalysts, of which the most important are substrate specificity, high catalytic activity, and the ability to work in moderate

environmental conditions (1). The current demand for better utilization of renewable resources and pressure on various industries to operate within environmentally compatible limits have stimulated development of new enzyme-based industrial processes. Alkaline proteases are extensively used in detergents, leather processing, feeds,

and the chemical industry, as well as in waste treatment (2). Global proteolytic enzyme demand for 2005 was expected to increase dramatically from 1.0 to 1.2 billions dollars because of wide-ranging applications in different industries (3). Proteases, most of which are alkaline proteases, constitute 60%-65% of the global industrial enzyme market (4).

Generally, proteases produced from microorganisms are constitutive or partially inducible in nature, and their production is mostly under the influence of culture conditions (5). *Bacillus* species are major producers of proteolytic enzymes, which are produced during post-exponential and stationary phases of growth. Extra-cellular 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 (6-8). 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 (9). In fact, the optimization of different process parameters and the quantity of medium ingredients are basic tools for making enzyme production economically feasible.

The aim of the present study was to investigate the effects of various fermentation parameters, such as growth medium pH, incubation temperature, and age and size of inoculum, on the yield of protease production by *B. licheniformis* N-2. Kinetic parameters were also evaluated to determine if the bacterial enzyme production was growth associated.

Materials and Methods

Microorganism

A strain of *Bacillus licheniformis* N-2 isolated from decaying organic soil was used for the production of alkaline protease (10). *B. licheniformis* N-2 was cultivated on nutrient agar slants by incubating at 37 °C for 24 h. The culture was then preserved at 4 °C and transferred to new slants every 30 days in order to keep them viable. The nutrient agar medium was composed of (g/l): Lab-Lemco powder, 1; yeast extract, 2; peptone, 5; sodium chloride, 5; agar, 15. The nutrient agar was adjusted to pH 10 with 1 N HCl/NaOH before sterilization at 121 °C for 15 min.

Preparation of Inoculum

Inoculum was prepared by transferring a loopful of 24-h-old *B. licheniformis* N-2 culture into 50 ml of inoculum medium of the following composition (g/l): glucose, 5; soybean meal, 10; K₂HPO₄, 5; MgSO₄·7H₂O, 0.5; NaCl, 0.5; CaCl₂·2H₂O, 0.5. The inoculated broth was incubated in a water bath shaker (Eyela, Japan) for 24 h at 37 °C for the propagation of bacteria of up to 10⁸⁻¹⁰ cells/ml. Then 1% (v/v) of this inoculum medium was used for the production of alkaline protease in the subsequent experiments.

Cultivation of Growth Medium

The initial medium used for alkaline protease production was composed of (g/l): glucose, 10; soybean meal, 10; K₂HPO₄, 5; MgSO₄·7H₂O, 0.5; NaCl, 0.5; CaCl₂·2H₂O, 0.5. The medium was adjusted to pH 10 with 1 N HCl/1 N NaOH before sterilization at 121 °C for 15 min. The growth medium was inoculated with 24-h-old culture broth (1% (v/v) of inoculum).

Optimization of Process Parameters

Optimization of the process parameters was conducted in order to evaluate the effect of each individual parameter separately and to incorporate it as a standard level before optimizing the next parameters.

Effect of Incubation Period

The effect of incubation period was studied by incubating the growth medium for 12, 24, 36, 48, 60, and 72 h. Enzyme activity was measured after each interval to determine the most suitable incubation period for maximum protease production by *B. licheniformis* N-2.

Effect of Initial pH

Optimum pH for the production of alkaline protease was determined by adjusting the initial pH of the growth medium to different levels (7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10, 10.5, and 11). The pH of each growth medium was adjusted with 1 N HCl/NaOH before sterilization at 121 °C for 15 min.

Effect of Cultivation Temperature

The effect of inoculation temperature on enzyme production was studied by incubating the growth medium at growth temperatures ranging from 25 to 50 °C. Enzyme activity and cell biomass were determined at each temperature.

Effect of Agitation Speed

Different agitation speeds (60, 80, 100, 120, 140, and 160 rpm) at pre-optimized conditions were studied to determine the best agitation speed for maximum yield of protease and growth of *B. licheniformis* N-2.

Effect of Age and Size of Inoculum

The effect of inocula age (6, 12, 18, 24, 30, 36, 42, and 48 h) and size (0.5%-7.0%) were evaluated for maximum proteolytic enzyme yield. Inoculum density was kept in the range of 10^{8-10} cells/ml for this purpose.

Determination of Kinetic Parameters

Different kinetic parameters for biomass and product yield were determined according to the methods described by Pirt (11), and Okpokwasili and Nweke (12). All these kinetic parameters were studied in optimized culture conditions in the batch fermentation process.

Analytical Procedures

Total Protein Content

Total protein content of the samples was determined by the method of Lowery, using bovine serum albumin (BSA) as the reference standard (13).

Total Glucose Estimation

Total glucose content was determined according to the phenol-sulfuric acid method described by Dubois et al. (14).

Total Biomass Determination

Biomass content was determined by measuring the dry weight of a known quantity of sample. The sample was then centrifuged at 90,000 g (Eppendorf 5804 R) for 10 min at 4.0 °C and washed with normal saline several times to remove the suspended soybean particles. The washed sample was dried at 105 °C to obtain constant weight.

Table. Kinetic parameters of growth and protease production for batch fermentation by *B. licheniformis* N-2 at optimized culture conditions in the production of medium at initial pH 10, temperature 37 °C, and agitation speed of 140 rpm in shake flask cultures.

Cultivation time (h)	Kinetic Analysis Parameters					
	μ (h^{-1})	$Y_{x/s}$ (g of cells/g)	q_s (g/g of cells per h)	$Y_{p/s}$ (U/g, 10^3)	$Y_{p/x}$ (U/g, 10^3)	q_p (U/g per h, 10^3)
0	0 ^k	0 ^f	0 ^g	0 ^h	0 ^g	0 ^j
4	0.054 ± 0.002 ^j	0.095 ± 0.003 ^e	0.005 ± 0.001 ^g	0 ^h	0 ^g	0 ^j
8	0.308 ± 0.006 ^a	0.166 ± 0.006 ^d	0.051 ± 0.006 ^b	40.44 ± 1.40 ^g	242.43 ± 5.80 ^e	74.67 ± 3.30 ^a
12	0.301 ± 0.010 ^a	0.267 ± 0.001 ^c	0.080 ± 0.003 ^a	48.69 ± 1.60 ^f	181.79 ± 4.20 ^f	54.72 ± 2.10 ^b
16	0.246 ± 0.004 ^b	0.288 ± 0.006 ^c	0.071 ± 0.001 ^b	50.65 ± 2.00 ^f	175.76 ± 6.10 ^f	43.24 ± 1.40 ^d
20	0.215 ± 0.003 ^c	0.383 ± 0.012 ^a	0.082 ± 0.011 ^a	69.22 ± 1.70 ^e	180.545.70 ^f	38.82 ± 1.02 ^e
24	0.178 ± 0.004 ^d	0.375 ± 0.013 ^a	0.067 ± 0.006 ^b	99.3 ± 2.30 ^a	264.28 ± 4.20 ^{ab}	47.04 ± 0.89 ^c
28	0.154 ± 0.005 ^e	0.374 ± 0.009 ^a	0.058 ± 0.002 ^c	98.23 ± 1.80 ^a	262.65 ± 3.40 ^a	40.45 ± 1.01 ^e
32	0.135 ± 0.003 ^f	0.373 ± 0.010 ^a	0.050 ± 0.001 ^d	97.09 ± 2.10 ^a	260.312.80 ^{bc}	35.14 ± 0.88 ^f
36	0.119 ± 0.009 ^g	0.371 ± 0.002 ^a	0.044 ± 0.001 ^{de}	93.67 ± 1.20 ^b	252.48 ± 6.20 ^{cd}	30.05 ± 1.02 ^g
40	0.107 ± 0.006 ^h	0.368 ± 0.008 ^a	0.039 ± 0.002 ^{ef}	88.22 ± 1.10 ^c	239.73 ± 2.80 ^{de}	25.650.68 ^h
44	0.097 ± 0.009 ⁱ	0.366 ± 0.020 ^a	0.035 ± 0.001 ^f	86.6 ± 1.60 ^{cd}	236.62 ± 3.10 ^e	22.95 ± 0.71 ⁱ
48	0.089 ± 0.003 ⁱ	0.362 ± 0.013 ^b	0.032 ± 0.001 ^f	84.15 ± 2.10 ^d	232.47 ± 4.80 ^e	20.69 ± 1.20 ⁱ

Each value in each column is an average of 3 parallel replicates ($\pm$ indicates SD among the replicates). Values followed by different letters differ significantly from each other at $P \leq 0.05$, based on CoStat software.

Abbreviations:

μ (h^{-1}): Specific growth rate.

$Y_{x/s}$: g of cell biomass/g of glucose consumed.

q_s : g of glucose consumed/g of cell biomass per h.

$Y_{p/x}$: Enzyme produced/g of cells formed.

$Y_{p/s}$: Enzyme produced/g of glucose consumed.

q_p : Enzyme produced/g of cells per h.

Determination of Proteolytic Activity

Alkaline protease activity was determined using casein as a substrate at a concentration of 1.0% in 0.05 M glycine-NaOH buffer at pH 11.0 (15). The substrate was treated with a known quantity of the enzyme for 15 min at 60 °C. One proteolytic unit (PU) was defined as the quantity of enzyme that released 1 µg of tyrosine/ml per min under the assay conditions.

Statistical Analysis

All the experiments were carried out independently (in triplicate) in 250-ml Erlenmeyer flasks. The data represented here are in the form of mean $\pm$ SD. Significance is presented as Duncan's multiple range test results in the form of probability ($P \leq 0.05$) values, which were obtained using CoStat 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 by *B. licheniformis* N-2. 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.

Effect of Incubation Period

In order to determine the optimum incubation time for maximum enzyme production, 50 ml of the growth medium was inoculated with 1% (v/v) of 24-h-old *B. licheniformis* N-2 culture. The inoculated flasks were treated for different time intervals at 37 °C and 140 rpm. Optimum enzyme production was obtained after 24 h of incubation (Figure 1). Thereafter, a rapid decline in enzyme activity was observed, 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. To determine which method to employ, one has to estimate the cost and consider extending the fermentation period; therefore, increasing the operational cost of any fermentation process would be compensated for by the extra yield of the required product. Maximum enzyme production was observed after 96 h with alkalophilic *Bacillus* sp. isolated from natural habitats (7). A broad incubation period ranging from 48 to 70 h for maximum protease enzyme yield by *Bacillus* strains has been previously reported (8,16). The main advantage of enzyme production by *B. licheniformis* N-2 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.

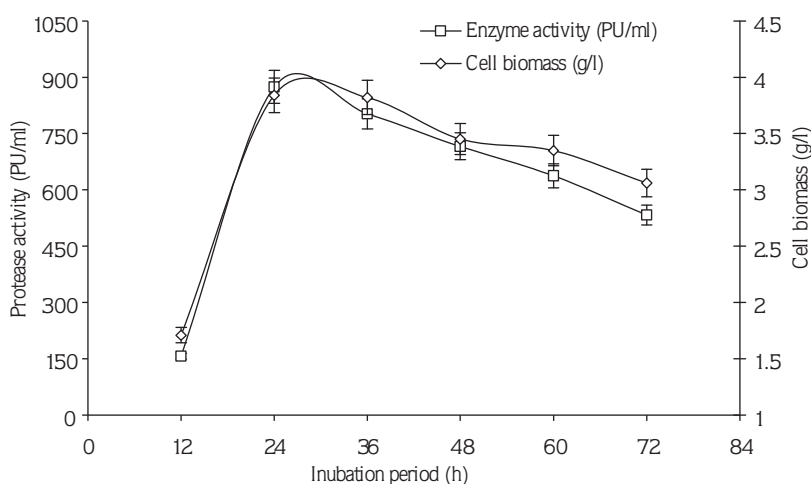


Figure 1. Effect of incubation period on growth and production of alkaline protease by *B. licheniformis* N-2 at an initial pH of 10, 37 °C, and agitation speed of 140 rpm in shake flask cultures.

Effect of Media pH

The effect of initial pH was investigated in order to determine the most suitable pH for the growth of *B. licheniformis* N-2 and alkaline protease production (Figure 2). The highest alkaline protease yield (875.44 PU/ml) was recorded at an initial growth medium pH of 10. These findings indicate that *B. licheniformis* N-2 is sensitive to change in pH because change in growth medium pH affects protease growth and 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 (2); therefore, it is concluded from the present

experimental results that adjustment of initial growth medium pH is necessary for maximum alkaline protease yield by *B. licheniformis* N-2. Maximum alkaline protease production has been reported in different pH ranges (7-10) for different *Bacillus* spp. (7,17). pH requirements vary from species to species and even in different stains of the same species isolated from different habitats.

Effect of Incubation Temperature

Temperature is one of the most critical parameters to be controlled in any bioprocess (18). The effect of temperature on alkaline protease production revealed that maximum yield was obtained at 37 °C (Figure 3). A

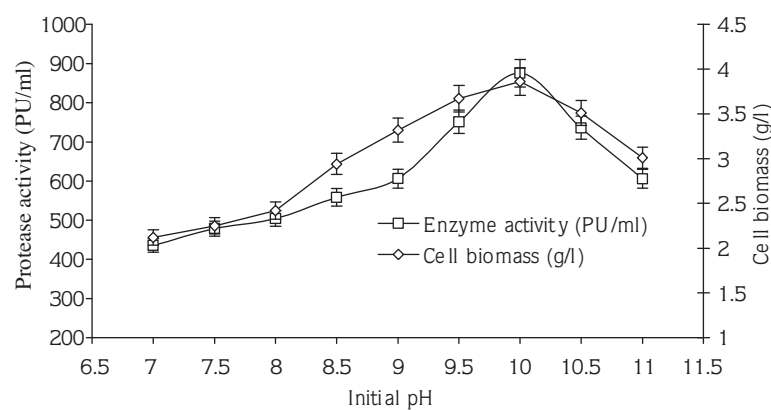


Figure 2. Effect of initial pH on the growth and alkaline protease production by *B. licheniformis* N-2 at an agitation speed of 140 rpm and 37 °C after 24 h of incubation in shake flask cultures.

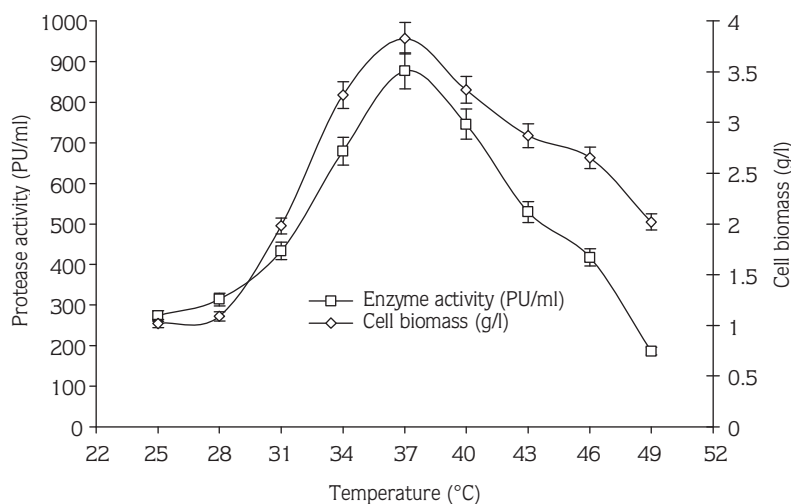


Figure 3. Effect of temperature on growth and alkaline protease production by *B. licheniformis* N-2, at initial pH 10 and agitation speed of 140 rpm after 24 h of incubation in shake flask cultures.

decrease in enzyme yield was observed with further increases in temperature; thusly, production of alkaline protease by *B. licheniformis* N-2 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 (6,19).

Effect of Agitation

The effect of different agitation speeds on the protease production and growth profile of *B. licheniformis* N-2 was observed by incubating the fermentation medium at pre-optimized conditions (Figure 4). The lowest alkaline protease activity (317.16 PU/ml) and cell growth (dry cell weight: 2.11 g/l) were observed at 40 rpm, suggesting the limitation of oxygenation as well as impaired homogenization of the medium components; however, alkaline protease production and growth of *B. licheniformis* N-2 increased as agitation speed increased. Based on the results, 140 rpm was determined to be the optimum agitation speed for maximum yield (879.31 PU/ml) and thereafter declines in protease activity and cell growth were observed at 160 rpm. It can therefore be suggested that sheer stress caused the disruption of the cells. All these findings indicate that oxygenation and uniform mixing of the medium ingredients impaired growth as well as

production of alkaline protease. Earlier investigations reported that 180 and 220 rpm are the most suitable agitation speeds for enzyme production by most *Bacillus* strains (20,21). Maximum protease activity was observed from alkalophilic *Bacillus* species at 100 rpm by Genekal and Tari (7). These slight variations might be due to differences in medium composition or culture conditions, which significantly affect growth and alkaline protease production of *B. licheniformis* N-2.

Effect of Age and Size of Inoculum

B. licheniformis N-2 inocula of different ages were used to inoculate the growth medium. Results indicated that 24-h-old *B. licheniformis* N-2 inoculum was the most suitable for protease biosynthesis during the fermentation process (Figure 5). Maximum enzyme production was observed with 2.0% inoculum size and further increases resulted in decreases in alkaline protease production (Figure 6). With higher inoculum concentrations the nutrient might have been consumed rapidly and overall resulted in less growth and alkaline protease production. These findings are in good agreement with the study by Kanekar et al. (22), who reported maximum enzyme activity (167.28 U/ml) with 2.0% inoculum size and 24-h-old *Bacillus alcalophilus* culture. Optimum protease activity (107 PU/ml) with 10% inoculum size and 24-h-old by *B. subtilis* PCSIR-5 was reported by Nadeem et al. (8). All these findings indicate that age and inoculum concentration have profound effects on cell growth and protease production, depending upon the characteristics of the strains.

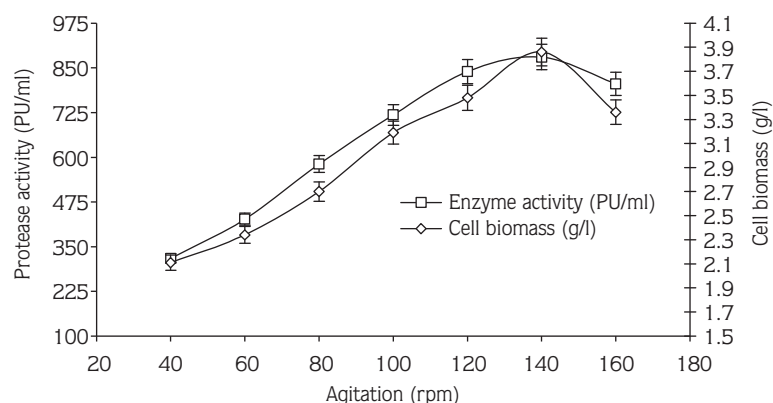


Figure 4. Effect of agitation speed on growth and production of alkaline protease by *B. licheniformis* N-2 at an initial pH of 10 and 37 °C after 24 h of incubation in shake flask cultures.

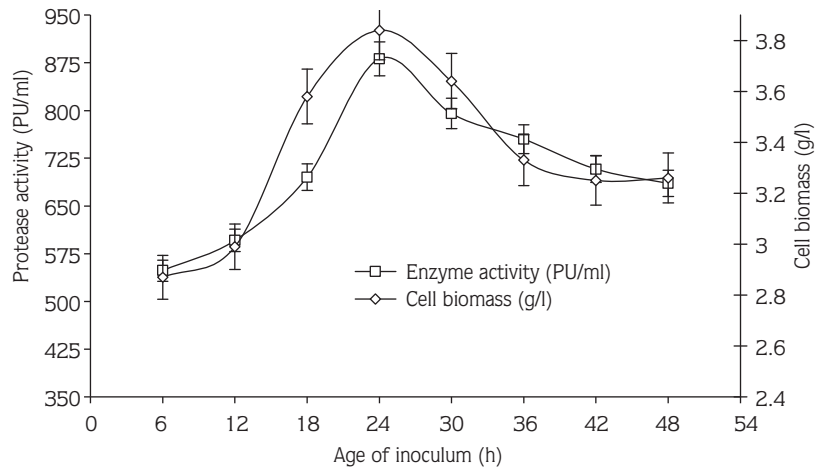


Figure 5. Effect of inoculum age on the growth and alkaline protease production by *B. licheniformis* N-2 at an initial pH of 10, agitation speed of 140 rpm, and 37 °C after 24 h of incubation in shake flask cultures.

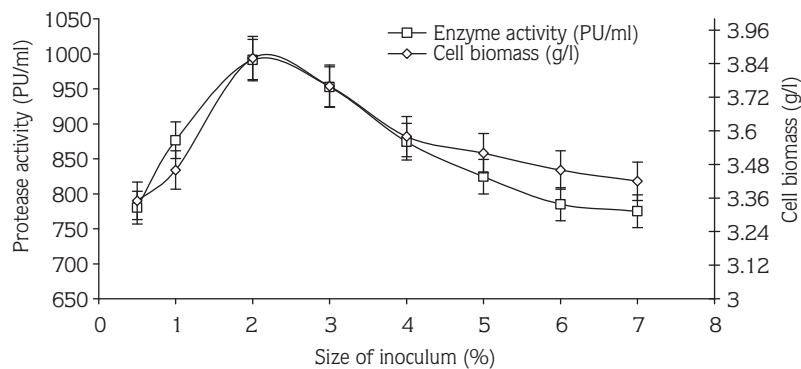


Figure 6. Effect of inoculum size on the growth and alkaline protease production by *B. licheniformis* N-2 at an initial pH of 10, agitation speed of 140 rpm, and 37 °C after 24 h of incubation in shake flask cultures.

Growth Kinetics Studies of *B. licheniformis* N-2 at Optimum Conditions

Results of batch alkaline protease fermentation by *B. licheniformis* N-2 on glucose, as a limited carbon source, in shake flask experiments are shown in Figure 7. Cell growth was limited during the first 4 h following inoculation and cell biomass increased from 0.05 g/l to 0.062 g/l, while substrate concentration decreased from 10 g/l to 9.35 g/l. This behavior is common in most fermentation processes in which a lag phase is required for adaptation to the new environment. The lag phase was followed by a log phase in which the substrate utilized decreased from 9.35 g/l to 0.02 g/l, while cell growth increased from 0.062 g/l to

3.75 g/l. The synthesis and secretion of alkaline protease were initiated during the exponential phase, with the maximum increase (991.04 PU/ml) at the end of the growth phase after 24 h of incubation. Subsequently, a stationary phase of almost constant biomass was observed over a period of 8 h; however, a retrogressive decrease in enzyme production was indicated after the exponential phase, which continued until the death phase. All these findings demonstrate that alkaline protease production by *B. licheniformis* N-2 was entirely growth-associated, and they also indicate that *B. licheniformis* N-2 is a fast-growing bacterium with a tremendous ability for adaptation to the fermentation medium.

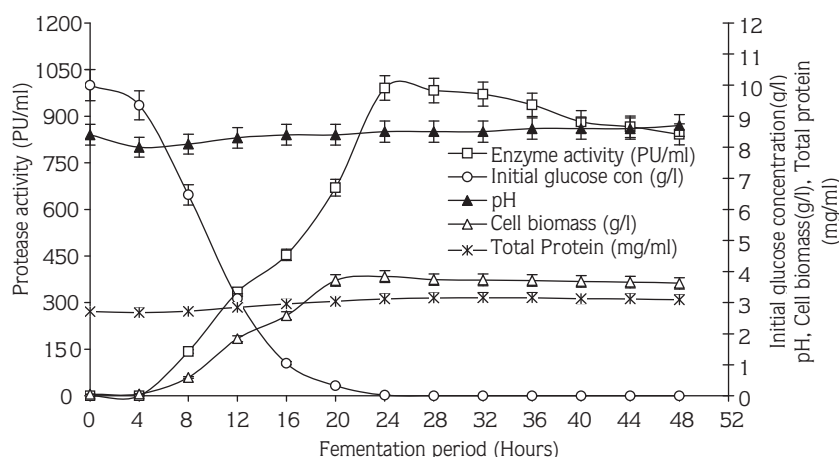


Figure 7. Kinetics studies of alkaline protease production by *B. licheniformis* N-2 at an initial pH of 10, agitation speed of 140 rpm, and 37 °C, in batch fermentation.

Biomass and product yield factors were estimated during the batch fermentation process (Table). Maximum $Y_{p/s}$ and $Y_{p/x}$ were 99.3 PU/g of glucose and 264.28 PU/g of cell biomass, respectively, after 24 h of incubation. These results indicate that enzyme production followed the growth of *B. licheniformis* N-2, suggesting that alkaline protease was produced as a primary metabolite; however, the maximum cell growth rate (0.308 h^{-1}) was observed 8 h after inoculation, which demonstrated that *B. licheniformis* N-2 grew faster during the initial stage of the exponential phase. A maximum growth rate (0.17 h^{-1}) by *Bacillus* sp. EL31410 after 6 h in optimized medium in batch fermentation was reported by He et al. (23). The biomass (0.023 g of cells/g of glucose) and product yield (0.021 U/g of glucose) for *Bacillus* L21 with a specific growth rate (0.7 h^{-1}) at optimized conditions was reported (7). These slight differences in growth rate and kinetic parameters between the previous reports and our findings might be due to differences in cultivation conditions as well as *Bacillus* species.

Conclusion

The results obtained in this study indicate that optimization of culture conditions played a central role in improving yield through the fermentation process. Maximum protease activity (991.04 PU/ml) was obtained after optimization of the fermentation process. It is well

known from kinetics studies that protease production is entirely growth-associated and there is a well-established balance between growth and product coefficients, which strongly indicate that *B. licheniformis* N-2 can be economically used for commercial alkaline protease production. The glucose utilization profiles show that glucose was completely utilized within 24 h of incubation, suggesting that further supplementation of growth ingredients through fed-batch fermentation under control conditions may improve the yield of alkaline protease by *B. licheniformis* N-2.

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References

1. Sandhya C, Nampoothiri KM, Pandey A. Microbial proteases, In: Barredo JL, ed. Microbial Enzymes and Bio-information. Human Press Inc., Totowa, New Jersey; 2005: pp. 165-180.
2. Kumar CG, Takagi H. Microbial alkaline proteases: from a bio-industrial view point. Biotechnol Adv 17: 561-594, 1999.
3. Godfrey T, West S. Introduction to Industrial Enzymology. In: Industrial Enzymology. 2nd Ed, Macmillan Publishers Inc, New York; 2001 pp. 3.
4. Banerjee UC, Sani RK, Azmi W et al. Thermo-stable alkaline protease from *Bacillus brevis* and its characterization as laundry detergents additive. Process Biochem 35: 213-219, 1999.
5. Beg QK, Saxena RK, Gupta R. De-repression and subsequent induction of protease synthesis by *Bacillus mojavensis* under fed batch operations. Process Biochem 37: 1103-1109, 2002.
6. Puri S, Beg QK, Gupta R. Optimization of alkaline protease production from *Bacillus* sp. by response surface methodology. Cur Microbiol 44: 286-290, 2002.
7. Genckal H, Tari C. Alkaline protease production from alkalophilic *Bacillus* sp. isolated from natural habitats. Enz Microb Technol 39: 703-710, 2006.
8. Nadeem M, Shahjahan B, Syed QA et al. Microbial production of alkaline proteases by locally isolated *Bacillus subtilis* PCSIR-5. Pak J Zool 38: 109-118, 2006.
9. Li Y, Lin J, Meng DJ et al. Effect of pH, cultivation time and substrate concentrations on the endoxylanase production by *A. awamori* ZH-26 under submerged fermentation using central composite rotary design. Food Technol Biotechnol 44: 473-477, 2006.
10. Nadeem M, Qazi JI, Baig S et al. Studies on commercially important alkaline protease from *Bacillus licheniformis* N-2 isolated from decaying organic soil. Turk J Biochem 32: 171-177, 2007.
11. Pirt SJ. Principles of Microbes and cell cultivation. In: Parameters of growth and analysis of growth data. Blackwell Scientific Publications, London, 1975: pp. 4-14.
12. Okpokwasili GC, Nweke CO. Microbial growth and substrate utilization kinetics. African J Biotechnol 5: 305-317, 2006.
13. Lowry OH, Roserbrough NJ, Farr AL et al. Protein measurement with folin phenol reagent, J Biological Chem 193: 265-275, 1951.
14. Dubois M, Gilles KA, Hamilton JK et al. Colorimetric method for determination of sugars and related substances. Anal Chem 28: 350-356, 1956.
15. Yang SS, Huang CI. Proteases production by Amyolytic fungi in solid state fermentation. J Chinese Agric Chem Soc 32: 589-601, 1994.
16. Denizci AA, Kazan D, Abeln ECA et al. Newly isolated *Bacillus clausii* GMBAE 42: an alkaline protease producer capable to grow under high alkaline conditions. J Appl Microbiol 96: 320-327, 2004.
17. Wellington CAN, Martins MLL. Production and properties of an extracellular protease from thermophilic *Bacillus* sp. Brazilian J Microbiol 35: 91-96, 2004.
18. Chi Z, Zhao S. Optimization of medium and cultivation conditions for pullulan production by new pullulan-producing yeast. Enz Microb Technol 33: 206-221, 2003.
19. Joo HS, Kumar CG, Park GC et al. Oxidant and SDS stable alkaline protease from *Bacillus clausii* 1-52: production and some properties. J Appl Microbiol 95: 267-72, 2003.
20. Joo HS, Kumar CG, Park GC et al. Optimization of the production of an extra cellular alkaline protease from *Bacillus horikoshii*. Process Biochem 38: 155-159, 2002.
21. Gupta R, Beg QK, Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 59: 15-32, 2002.
22. Kanekar PP, Nilegaonkar SS, Sarnaik SS et al. Optimization of protease activity of alkalophilic bacteria isolated from an alkaline lake in India. Biores Technol 85: 87-93, 2002.
23. He G, Chen Q, Ju X et al. Improved elastase production by *Bacillus* sp. EL31410 - further optimization and kinetics studies of culture medium for batch fermentation. J. Zhejiang Univ Sci 5: 149-156, 2004.