African Journal of Biotechnology Vol. 11(23), pp. 6335-6344, 20 March, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB09.1017
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Examining kinetic parameters of fermentation process in batch cultures on the production of alkaline protease

M.T. Hamed Mosavian[1], J. Sargolzaei[1], F. Tabandeh[2], H. R. Hosseinian Moghaddam[2] And B. Yakhchali[2]

[1]Ferdowsi University of Mashhad, Chemical Engineering Department, Mashhad.
[2]Industrial and Environmental Biotechnology Department, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran.

Accepted 30 December, 2010

Alkaline proteases are important group of enzymes because of their use in many industrial applications such as in detergent materials for the removal of the protein stains on clothes. The aim of this study is to investigate the kinetic parameters of the fermentation process inspected for alkaline protease production by using a local *Bacillus clausii*. In this way, it is carried out under fed-batch operations of fermentation in erlyne. Designing full factorial is used for optimizing appetite composition for the three factors of sponge carbon glucose, yeast extract (organic nitrogen), salt solution (including K_2HPO_4, $MgSO_4.7H_2O$), and trace element solution (TES) each at two levels and designed by eight tests in total. All experiments were carried out in duplicates to decrease the possibility of errors occurring in the research. Analysis of variance (ANOVA) showed that the high coefficient of the determination (R^2) value is 0.99, thus ensuring a significant adjustment of the suggested polynomial model with the experimental data. The results showed that protease production was controlled because of catabolic inhibition in the initial glucose concentration and the amount of enzyme activity which is important for protease production which increased in the end of the stationary phase that was often accompanied by sporulation phenomena. The results on comparison and inspection in batch cultures with glucose demonstrated that different values revealed that the alkaline protease was highly produced in a few hours after complete consumption of glucose. Also, mineral salts and yeast extract induce the protease production as increasing protease is a changing approach of bacteria catabolitic from development to extra cell enzyme production. Meanwhile, results showed that the detergent type do not have any effect on protease activity. Therefore, the production enzyme can be used as detergent powders.

Key words: Alkaline protease, *Bacillus clausii*, fed-batch fermentation, sporulation, kinetic parameters, full factorial, detergent.

INTRODUCTION

Microbial proteases are important enzymes of hydrolysis in which many researches are carried out on the basis of biochemistry properties, application, and their products.

These enzymes have an important role in metabolic processes and they have been considered for industries (Kalisz, 1988). Today, about sixty percent of trade enzymes concerns proteases (Godfrey and West, 1996). Considering trade aspects, proteases have different applications in leather and food industries as most applications of protease are of concern to detergent industries especially active and steady proteases in alkaline conditions (Gupta et al., 2002a, b). Alkaline protease production of extra cell is due to batch culture combination such as carbon and nitrogen sources, through the

*Corresponding author. E-mail: mosavian@um.ac.ir or hmosavian@gmail.com.

Abbreviations: PEG, Polyethylene glycol; **ATPase,** aqueous two phase systems; **TSoo,** dextrin; **C,** carbon; **N,** nitrogen; **Rpm,** round per minute; **OD,** optical density; **OD_{600},** optical density in wave length 600 nm; **TCA,** tyrosine concentration absorption; **Abs,** optical absorption; **Abs_{275},** optical absorption solution in wave length 275 nm; **U,** unit; **TES,** trace element solution.

presence of carbohydrates with light metabolizes similar to glucose (Beg et al., 2002a; Gupta et al., 2002a; Kole et al., 1988), and the presence of metal ions (Varela et al., 1996). Also, other agents affect the amount of protease production such as aeration, inoculation leaven density, pH, temperature, and incubation time (Hameed et al., 1999; Nehete et al., 1985). Alkaline protease first started the stationary phase that is usually simultaneous with the conversion of cellular growth to spore (Godfrey and West, 1996). Industrial protease was usually produced in fed-batch fermentation with very low special growth intensity (Kalisz, 1988). Optimization of the broth composites caused equilibrium in different materials and the materials used at fermentation at the end of experiment (Adinarayana and Ellaiah, 2002). Using statistical designs for optimization of bacteria culture conditions and enzyme production caused fast differentiation of influence agents (Pinar-Calik et al., 2003). It is observed that complex carbon and nitrogen sources can be used for industrial production of alkaline protease by Bacillus (Uyar and Baysal, 2004). In 1960, the Askalaz detergent was made from *Bacillus licheniformis* by NOVO Company with the trademark “Biotex”. Beshay could increase protease activity 3.11 times with bacteria stabilized on calcium Alginate (Beshay, 2003). Other methods have been used in protease production from different microorganisms such as the cell stabilization of different bacteria (Landau et al., 1995), an aqueous two phase systems (ATPase) consisting of polyethylene glycol (PEG) and potassium phosphate by *Bacillus thuringiensis* (Hotha and Banik, 1997). ATPase system consists of PEG and Dextron (TSoo) by *B. licheniformis* (Lee and Chang, 1990), and solid culture fermentation. In this research, kinetic parameters have been determined in two phases of bacterial growth and protease production and their quality of attentions have been considered by using the batch fermentation process.

Theory

Proteases

The proteases organize an enormous part of enzyme collections that differ from each other in properties of substrate, active site and catalytic mechanism, pH, thermal activity, and stability. These enzymes rule in every cell in processes such as protein recovery, cell nutrition, sporulation, budding of spore, and enzymes discharge. Proteases have variant activities on the basis of arrangement of an enzyme active area and revenue method. Proteases have been divided into three groups of acidic, alkaline, and inert proteases according to activity in different pH values in which each one of this pH limit is produced by special bacteria and is applied in various industries. Protease generation is an inherent ability of all microorganisms. In spite of the high microbial sources for making proteases, a few of them have been recognized to trade producers (Gupta et al., 2002b). Another important condition for a protease is enzyme steady in high pH and temperature for application in detergents.

Effective factors on alkaline protease production

Extra cell proteases was produced at the end of exponential and stationary phase by *Bacillus*. In microorganisms, an extra cell protease secretion induced by broth composite and/or concentration of carbon and nitrogen (C/N), some carbohydrate present such as glucose, metal ions and amino acids as a nitrogen source. Furthermore, the production of protease could be controlled by manipulation of physical factors like aeration, cellular compress of inoculation leaven, pH, incubation temperature and time (Beg et al., 2002b; Gupta et al., 2002b; Lee et al., 1996; Puri et al., 2002). Organic nitrogen sources are substrate and inducers of protease production like aligopeptide, casamino acid, casein, yeast extract and soy flour (Beg et al., 2002a; Chu et al., 1992; Gupta et al., 2002b; Pinar-Calik et al., 2003)

Fermentation process in batch culture

The batch culture strategy is a process in which a box which contains an amount of broth initially with nutrition material is added or excited. In this broth, with the reduction of substrate and coagulation of metabolites, the rate exponential phase is completed and microorganisms are exerted to the stationary phase and are dealt with subsequently. This type of culture is usually used in laboratories and industries.

MATERIALS AND METHODS

Bacteria genealogy alkaline protease material

The bacterium used in this research was *B. licheniformis* L2 which was isolated from samples of soil; subsequently the activity of protease was established. Then, it was distinguished from *Bacillus clausii* by complementary experimental and series assign of 16SrRNA.

Culture conditions and bacteria maintenance

According to the application of alkaline proteases in different industries, like detergents, the fermentation process was used to produce alkaline protease. Producing extra cell protease is the limitation of food materials for bacteria in the beginning period of the stationary phase. So in this investigation, by non-continuous fermentation, alkaline protease enzyme was produced using Bacillus bacteria. By using statistical methods, feeding combinations were optimized.

The medium was used for preparation of stock culture and maintenance of bacteria at -70°C with combination of mixed 1% peptone and 30% glycerol. These cultures were incubated at 35°C and then were refrigerated in a maximum time of one week.

Table 1. Composites of broth with different carbon sources.

Combinational material	(g/l)
Trisodium citrate	4
K_2HPO_4	4
Yeast extract	7
KNO_3	5
Na_2CO_3	10
$MgSO_4.7H_2O$	0.5
$CaCl_2$	0.002
Trace element solution	10ml

Table 2. Concentration of rare metal elements of ionic solution in broth.

Trace element solution	(g/l)
Trisodium citrate	10
$(NH_4)_6MO_7O_{24}$	0.1
$MnSO_4.H_2O$	0.5
$FeSO_4.7H_2O$	2
$CuSO_4.5H_2O$	0.2
$ZnCl_2$	0.2

Alkaline broth

The liquid broth was used for bacteria growth curve and protease production. Alkaline broth prepared consisted of glucose (10 gl^{-1}), peptone (5 gl^{-1}), yeast extract (5 gl^{-1}), KH_2PO_4 (1 gl^{-1}), $MgSO_4.7H_2O$ (0.2 gl^{-1}) and Na_2CO_3 (10 gl^{-1}).

The medium pH was adjusted to less than 10.5. A solution of $MgSO_4.7H_2O$ and Na_2CO_3 was sterilized separately in 121°C for 15 min using an autoclave, and then was added to the medium. Furthermore, the solid alkaline broth was made with the addition of 1.5 (% W) agars.

Broth with different carbon sources

The broth contains 10, 15 and 20 gl^{-1} of glucose, and 10 gl^{-1} sucrose has been used to inspect the bacteria growth and alkaline protease production in the presence of different glucose and sucrose sources in a semi identify broth as shown in Table 1. The medium pH was 10.5.

The rare metal elements in ionic solution are described in Table 2. In all mediums containing glucose, a solution of $MgSO_4.7H_2O$, Na_2CO_3 and $CaCl_2$ was sterilized separately in 121°C for 15 min in an autoclave and then added to other composites of broth after cooled.

The solution of rare metal elements was sterilized by filters of 0.45 µm mesh, and 10 ml of this solution was added to medium each of 1 ml broth. The pH of the medium was increased to 10 with sterile Na_2Co_3 solution.

Growth conditions

The microorganism used in this study was a local species of Bacillus bacteria. The first and second cultures were used for the bacteria cells' agreement.

Table 3. Concentration of the material in broth.

Combination materials	(g/l)
Trisodium citrate	4
K_2HPO_4	4
Yeast extract	7
KNO_3	5.8
Na_2CO_3	10
Glucose	5
$MgSO_4.7H_2O$	0.5
$CaCl_2$	0.002
Trace element solution	10 ml

The 50 ml of the first culture was prepared to 250 ml Erlenmeyer flask and the 100 ml of second culture with composites of Table 3 in two 500 ml Erlenmeyer flasks. A single colony was transferred to first culture from alkaline solid culture for 24 to 48 h. After 20 h at 35°C incubation with agitation speed of 300 rpm, the optical density was measured at wave length 600 nm and the colony inoculated to second culture of 1 to 10 (10%) with OD_{600} = 1.5.

Feed combination in batch process

Designing full factorial statistical method is used for optimizing appetite composition for the three factors of carbon source (glucose), organic nitrogen (yeast extract), and salt solution in feed for alkaline protease production each two levels which is totally designed in eight test as shown in Table 4 .

Protease activation quantity test

For the measurement of enzyme activation, the solution made with the reaction containing 1% 0.1 Molar Tris-base buffer, with pH = 9 was used, and in order to stop the enzyme activation and to make casein deposition, 10% three-chloro acetic acid as staler solution was used (Joo et al., 2002).

Bacteria growth curve and protease production in Erlyne

Five percent as inoculation leaven was transferred to 50 ml broth in 250 ml Erlyne. A magnet was used which is equivalent to 350 rpm rate at 35°C for aerating. At different time and sterile conditions, 2 ml of sample was removed each time and the growth amount was measured with OD_{600} nm and also, enzyme activation with studying released nitrozin from casein substrate at 275 nm wave lengths was reported.

In this way, the cultures were centrifuged for 15 min in 10000 g at 4°C and the activity was inspected by a quantity test. The growth curve and enzyme production curve and the time of maximum production of enzyme were determined.

Detergent's influence on protease activation

Enzyme activation in the presence of Sepid industrial detergent product of Paksan Company with three enzymes and Taj, product of Behdad Company with four enzymes was studied. At first, blue and red granulations (enzymes) were separated. For modeling washing conditions, detergent 7 gl^{-1} coarseness was prepared.

Table 4. Feed compound in batch process.

Test Number	Glucose (g/l)	Yeast extract (g/l)	Salt solution K_2HPO_4:$MgSO_4$:TES (g/l:g/l:ml/l)
1	10	100	10:1:1
2	10	100	50:5:5
3	10	50	10:1:1
4	10	50	50:5:5
5	30	100	10:1:1
6	30	100	50:5:5
7	30	50	10:1:1
8	30	50	50:5:5

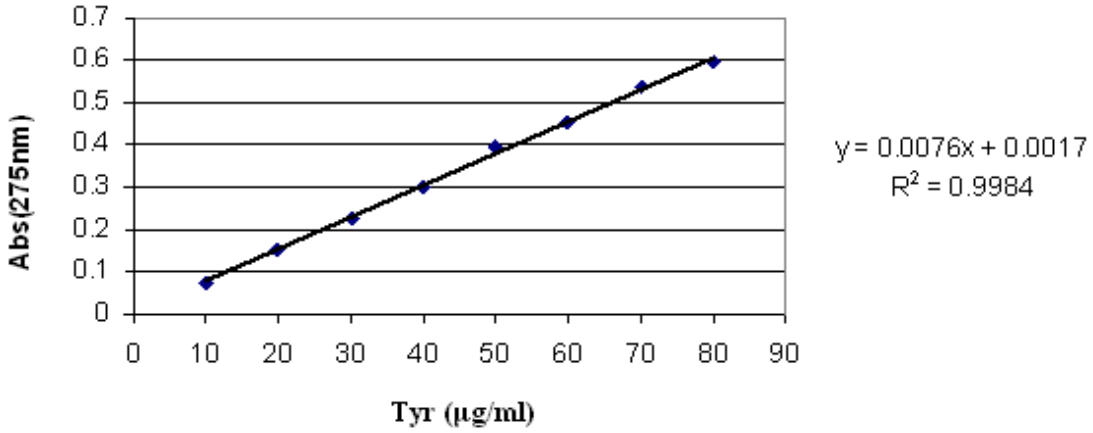

Figure 1. Tyrosine standard curve.

Then 1 ml enzyme (floating on broth) with 4 ml of solution (with coarseness of 7 gl^{-1} detergent) was incubated at 40°C for 10 to 20 min time intervals. After this duration, remaining enzyme activation was compared with whiteness without detergent and was reported in the figure of percentage.

Glucose measurement

The amount of glucose as a main carbon source in extract medium 2 to 3 h intervals using enzyme kit (Shim-enzyme Company Tehran-Iran) according to manufacture's structure was measured.

Statistical method

Full factorial method was used to study three factors' influence at two levels at the time as feed combination in batch extract to achieve the best feed combination for producing protease. All statistical analysis was done by Design-Expert 6.0.10 software made by stat-ease Company.

RESULTS AND DISCUSSION

In this investigation, Bacillus bacterium was separated from soil, identified and protease activation in it was confirmed and was named as *Bacillus licheniformis* L2. Then attending to broth role to increase alkaline protease enzyme production, different statistical methods for optimizing feed combination in broth were used, and some of the enzyme properties were marked.

With series assignment of 16 Sr RNA bacteria, it was defined that soil separated produce was *Bacillus clausii*. In this investigation, bacterial growth and alkaline protease enzyme with different amounts of glucose in erlyne were studied. Kinetic parameters in different extract conditions were determined and the results were compared and discussed as given below.

Determination of enzyme activity

To determine enzyme activity, light absorption amount for standard solutions containing different coarseness of tyrosine at 10% TCA and 275 nm wave length was measured and the standard curve was drawn (Figure 1).

According to tyrosine standard curve, this equation is given as:

$$C = (Abs_{275} - 0.0017) / 0.0076$$

Since C is tyrosine crassness by milliliter, Abs_{275} is light absorption of solution at 275 nm wave length and 0.0076

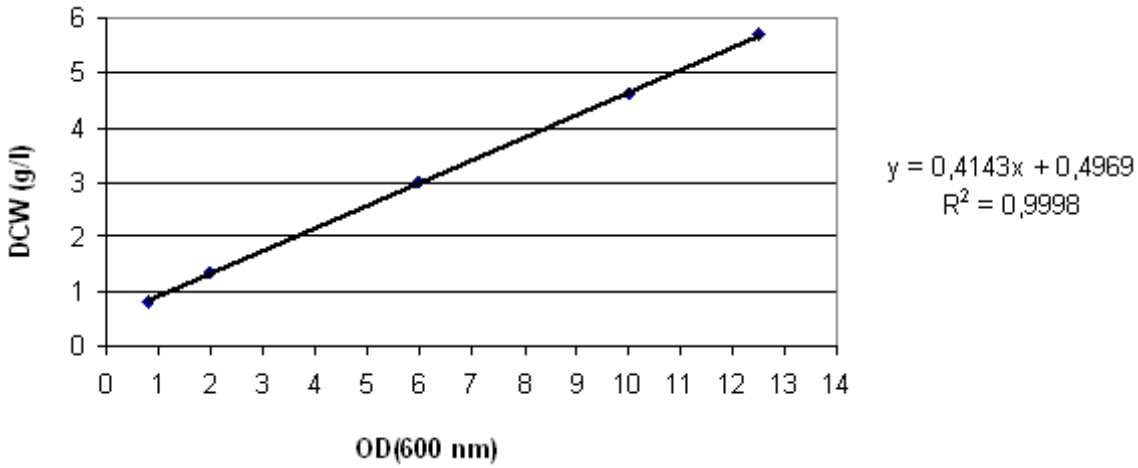

Figure 2. Dry cell weight proportion to wave length OD_{600} nm.

Table 5. Agents and their levels in feed combination.

Level (+1)	Level (-1)	Sign	Feed combination materials
30	10	X1	Glucose
100	50	X2	Yeast extract
50:5:5	10:1:1	X3	Salt solution

Table 6. Full factorial eight test composition and experimental correspond and predicted by software.

Predicted enzyme activity (U/ml)	Experimental enzyme U/ml)	X3	X2	X1	Test number
794.63	794±9.34	-1	-1	-1	1
850.38	851±9.34	+1	-1	-1	2
949.38	958±9.34	-1	+1	-1	3
845.63	845±9.34	+1	+1	-1	4
700.38	701±9.34	-1	-1	+1	5
831.62	831±9.34	+1	-1	+1	6
911.63	911±9.34	-1	+1	+1	7
883.38	884±9.34	+1	+1	+1	8

is the line slope.

Determination of drying bacteria weight

Dry cell weight and optical absorption increase at 600 nm in optimized broth and growth phase were conformed and the correlation between dry cell weight and OD_{600} was confirmed (Figure 2).

Optimization of feed combination at erlyne surface

For achieving the results faster and distinguishing the best feed, three factors: carbon source, organic nitrogen source and salt solution each at two levels were considered and at erlyne scale, their influences were studied. For this pulse, feeding was used in stationary phase. Then after reducing the glucose amount to less than 2 gl^{-1} feed combination was used. Feed combination was studied at a low level (-1) and a high level (+1) material combination or their conductions in feed was designed, so conduction of following materials are under inhibitor limit. Therefore, these amounts were considered for conduction in broth (Table 5).

Feed pulsing and with the amount l of 5 ml was added to broth and every 2 h sampling was done to measure the glucose conduction, enzyme activity and pH cell growth.

To study each factor influence and against effects, 3 factors in 2 levels complete factorial method ($2^3 = 8$) was used (Table 6). Each experiment was repeated twice.

Variance results analysis for carbon (glucose), organic

Table 7. Variance analysis (ANOVA) of feed combination in full factorial method.

Probe > F	F-valve	Freedom degree	Square sum	Source
0.0282	510.76	1	1596.13	X_1
0.0077	6822.76	1	21321.12	X_2
0.0577	121.00	1	378.12	X_3
0.0282	510.76	1	1596.13	X_1X_3
0.0211	912.04	1	2850.13	X_1X_2
0.0100	4070.44	1	12720.12	X_2X_3

Table 8. Variance analysis (ANOVA) for model.

Probe > F	F-valve	Freedom degree	Square sum	Changes Source
0.0165	2157.96	6	40461.75	Model
		1	3.12	Residual
		7	40464.88	Sum

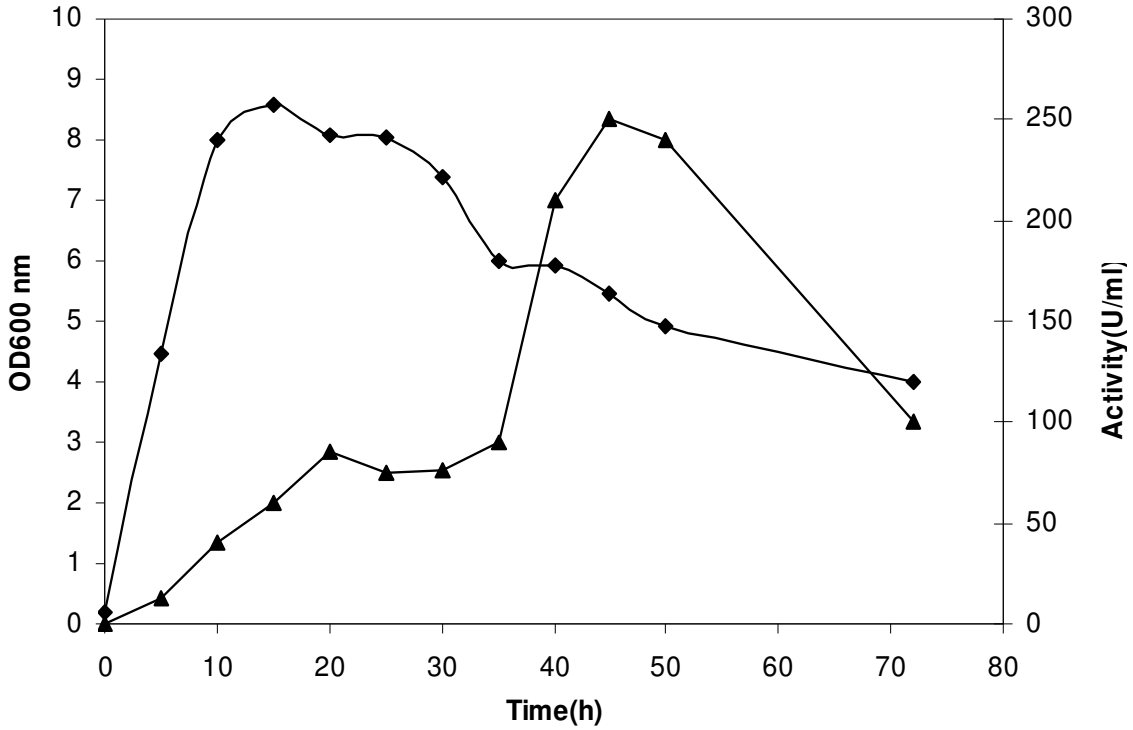

Figure 3. Bacteria growth curve (•) and protease production (U/ml) (▲) in alkaline broth at 35°C and agitation speed 350 rpm.

nitrogen source (extract yeast) and salt solution were achieved, by using statistical software (Table 7).

According to suggested levels, it is predicted that amount of protease production is 949.375 U/ml and its variations limit with 95% probable is between 920 and 980 with 1.65 standard average errors. Table 8 shows optimum conditions for feed combination and the predicted amount.

Bacterial growth and protease production in alkaline broth

The result of clausii Bacillus bacteria and protease production in alkaline broth is shown in Figure 3.

Bacterial growth was distinguished with optical absorption of samples at different times at 600 nm length and protease activity of samples with measuring the amount

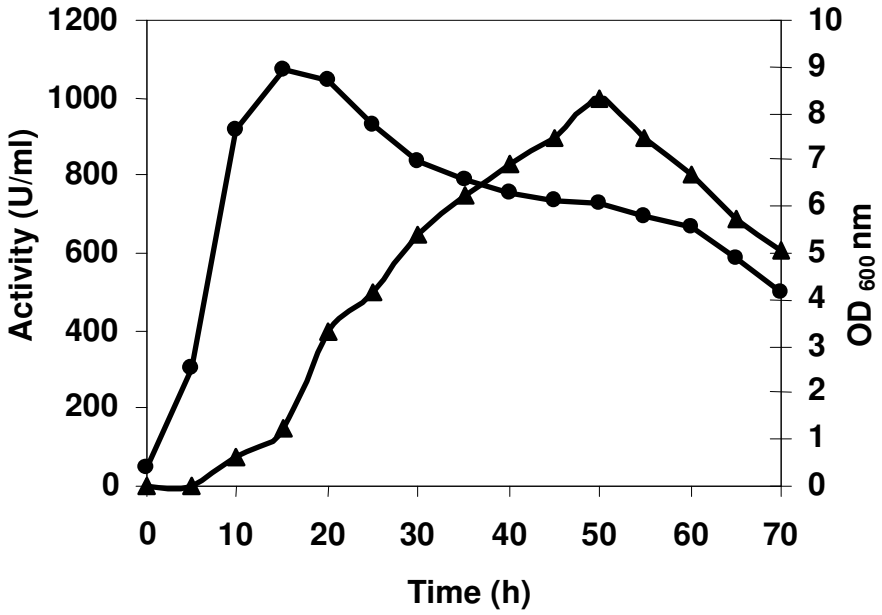

Figure 4. Bacteria growth curve (•) and protease production (U/ml) (▲) in broth containing sucrose $10\ \mathrm{gl}^{-1}$ at zero to 70 h time interval at 35°C and agitation speed 350 rpm.

of released tyrosine from hydrolyzing protease substrate based on absorption in 275 nm wave length. The study of bacterial growth in alkaline broth showed that exponential growth from bacteria inoculation time continued for almost 12 h. After this duration, bacteria had entered a stationary phase for 15 h. Then optical absorption decreased and after 48 h, optical absorption had been decreased by 50%.

It is shown that after 5 h bacteria inoculation to broth, producing enzyme started and did not increase considerably till 35 h. After this duration, producing enzyme strongly increased and after 48 h, it reached its maximum amount.

This activity rate remained for 10 h and decreased. Comparing growth curve and protease production proved that protease amount increased at the end of the exponential phase and the beginning of the stationary phase. Lastly, parts of stationary phase and beginning of dead phase have the maximum value. Therefore, protease is a production almost depending on growth. The maximum amount of producing protease in this medium with this condition is 250 U/ml. According to the mentioned condition, the maximum amount of protease was obtained after 48 h of incubation.

Bacteria growth and producing protease in broth containing sucrose

Bacterial growth and producing alkaline protease in optimized broth for producing protease which the main carbon source is sucrose was studied (Figure 4).

Results illustrated that the maximum amount of producing protease is obtained after 50 h incubation at 35°C with amount of $1000\ \mathrm{U}/\mathrm{ml}$ comparing bacteria growth which showed that in this broth, no more cell pile is obtained but the conditions for producing protease is more favorable. The proportion to alkaline broth protease production increased four times more.

Bacterial growth and protease production in broths with different amounts of glucose

Glucose is a mono-sacharide with a simpler structure than sucrose. It is also absorbed and used faster by cell.

In comparison to sucrose as a carbon source, which shows considerable reduction in protease production, Glucose did not show any reduction.

Faster measurements of glucose can facilitate the control of the process and hence better understanding of the amount of substrate is required for fermentation.

The curves of bacterial growth results, producing alkaline protease and consuming substrate in broths with different amounts of glucose, measuring optical absorption at 600 nm wave length and the protease activity by quantity test of protease activity based on optical absorption of substrate analysis done by enzyme at 275 nm wave length, the amount of substrate using an enzyme kit and also measuring optical absorption at 500 nm in mentioned broth in the materials and methods section

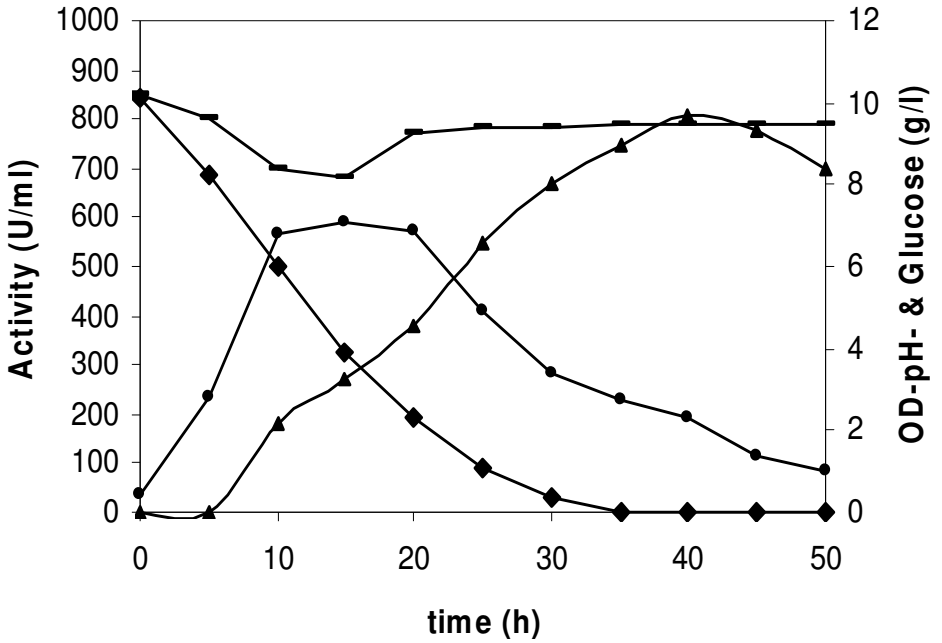

Figure 5. Bacteria growth (OD_{600}) (•), protease production (U/ml) (▲), glucose concentration (gl^{-1}) (♦) and pH-stat (-) in broth consist of glucose 10 gl^{-1} with agitation speed 350 rpm at 35°C.

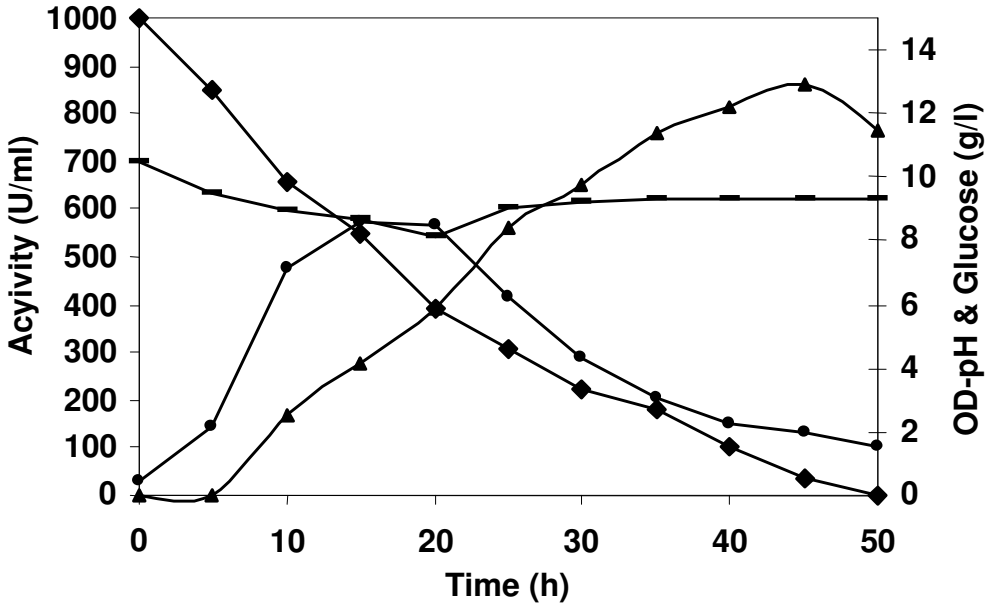

Figure 6. Bacteria growth (OD_{600}) (•), protease production (U/ml) (▲), glucose concentration (gl^{-1}) (♦), and pH-stat (-) in broth consist of glucose 15 gl^{-1} with agitation speed 350 rpm at 35°C.

have been presented in Figures 5 to 7. At several hours, sample's pH was measured by pH meter.

Results showed the substrates condense did not have much influence on alkaline protease activity which causes bacteria.

Table 9 shows the duration of fermentation, alkaline protease maximum activity and the amount of efficiency of mentioned broth.

By adding the condense of the initial glucose, the efficiency of producing protease reduces. In other words, consuming the substrate completely in lower condense of glucose as a main source for carbon causes the process

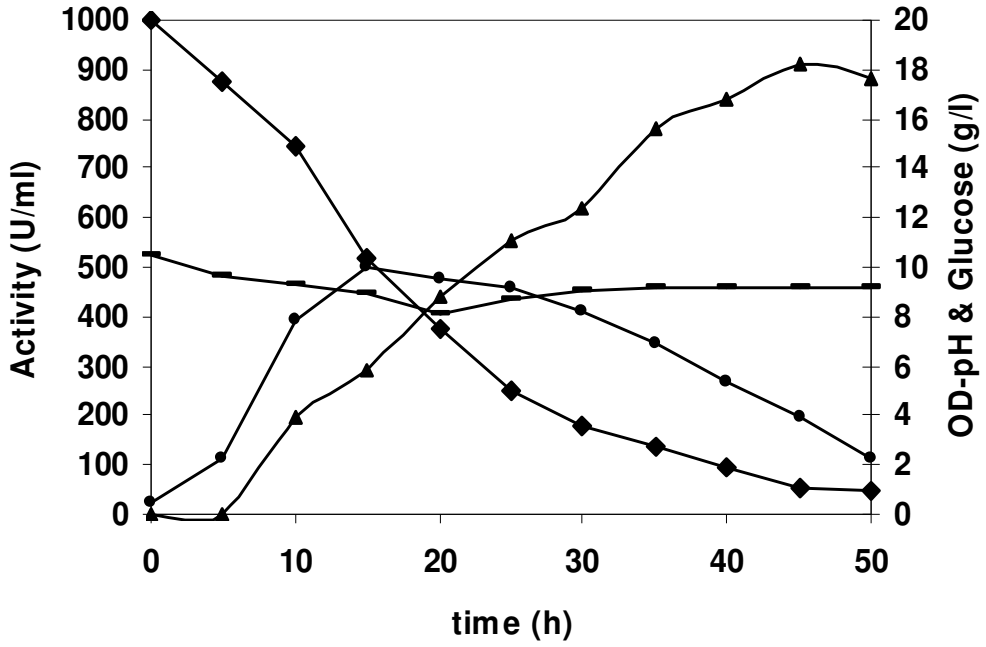

Figure 7. Bacteria growth (OD_{600}) (•), protease production (U/ml) (▲), glucose concentration (gl^{-1}) (♦), and pH-stat (-) in broth consist of glucose 20 gl^{-1} with agitation speed 350 rpm at 35°C.

Table 9. Examining and comparison of kinetic parameters of fermentation process in batch cultures in broths with different glucose amounts in erlyne with agitation speed 350 rmp at 35°C.

Gain 9U/l.h)	Efficiency (U/g)	Maximum enzyme activity	Maximum OD600nm	Fermentation time (h)	Initial glucose concentration	Kinetic parameter/ medium Number
20175	80700	807	7	40	10	1
19089	57267	859	8.2	45	15	2
20180	48119	9.8	10	45	20	3

efficiency to rise.

pH-stat and specified growth rate

During 10 to 12 h from fermentation time, the pH reaches from 10.5 to 8 which shows that the bacteria are at the end of exponential growth. Then pH of broth increases consequently, and almost after 27 h fermentation will get to 9. It seems that decreasing pH of broth is due to the production of acid by consuming glucose during the growth phase and when the rate of growth decreases pH starts to increase. This pH-stat during fermentation process can be a very good standard indicator for the time of beginning and ending of producing protease (Figure 8).

Conclusion

The results of bacterial growth and producing protease in alkaline broth illustrated that the amount of enzyme activity which is standard for producing protease increased at the end of the stationary phase, near the end of the stationary phase and in the beginning of the dead phase has attained to maximum amount (50 U/ml) which is often in company with sporulation phenomena. Subsequently, the activity of protease decelerates which is due to stopping enzyme synthesis, inactivating existing enzymes for the reason of complicated mechanism of outolise, inversion transformation of existing proteases, and also as a result of other protolitic of proteases attack. Examining and comparing the obtained results from broths with different amounts of glucose indicates that substrate concentration has no much influence on alkaline protease obtained from bacteria. Cell growth increases with increasing glucose concentration, whereas considerable changes have not been observed in producing protease. This case can be accused for harnessing effect of glucose on producing protease. Moreover, results indicated that the kind of detergent has no index effect on protease activity and it is because of the same combination of detergents. Since 75% of enzyme activity is remained after 10 min at 40°C, then

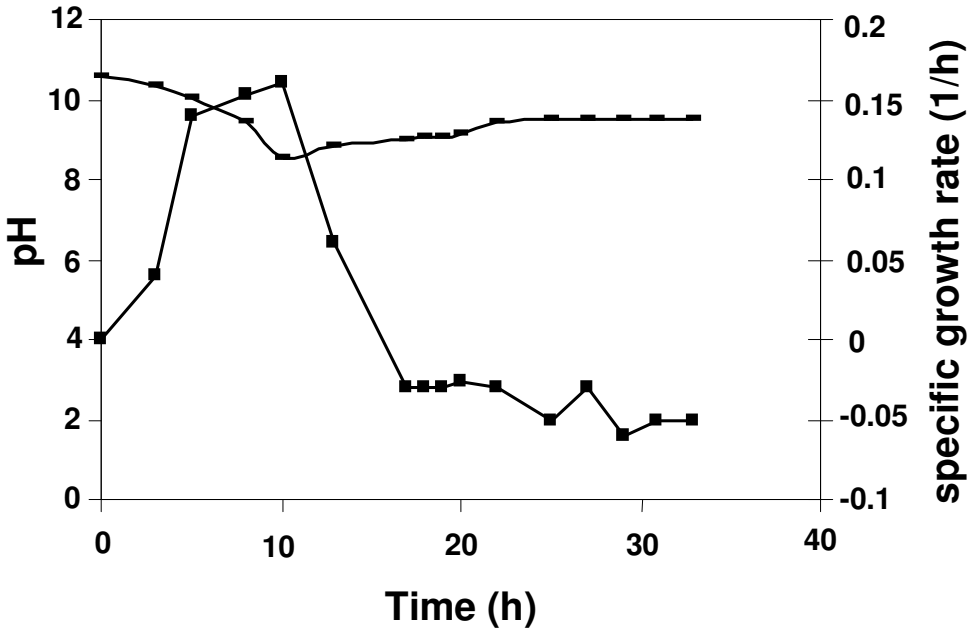

Figure 8. pH-stat and specified growth rate during batch fermentation process.

use of enzyme is possible in detergent powders.

REFERENCES

Adinarayana K, Ellaiah P (2002). Responce surface optimization of the critical medium components for the production of alkaline protease by a newly isolated *Bacillus* sp. J. Pharmaceut. Sci. 5(3): 272-278.

Beg QK, Saxena RK, Gupta R (2002a). De-repression and subsequent induction of protease synthesis by *Bacillus mojavensis* under fed-batch operations. Process Biochem. 37:1103-1109.

Beg QK, Saxena RK, Gupta R (2002b). Kinetic constants determination for an alkaline protease from *Bacillus mojavensis* using response surface methodology. Biotechnol. Bioeng. 78:289-295.

Beshay U (2003). Production of alkaline protease by Teredinobacter turnirae cells immobilized in Ca-alginate beads. Afr. J. Biotechnol. 2: 60-65.

Chu M, Lee C, Li T (1992). Production and degradation of alkaline protease in batch cultures of *Bacillus subtilis* ATCC 14416. Enzyme Microb. Technol. 14:755-761.

Godfrey T, West S (1996). Introduction to Industrial Enzymology. pp. 1-8.

Gupta R, Beg QK, Khan S, Chauhan B (2002a). An overview on fermentation, downstream processing and properties of microbial alkaline proteases. Appl. Microbiol. Biotechnol. 60: 381-395.

Gupta R, Beg QK, Lorenz P (2002b). Bacterial alkaline proteases: molecular approaches and industrial applications. Appl. Microbiol. Biotechnol. 59: 15-32.

Hameed A, Keshavarz T, Evans CS (1999). Effect of dissolved oxygen tension and pH on the production of extracellular protease from a new isolate of *Bacillus subtilis* K2, for use in leather processing. J. Chem. Technol. Biotechnol. 74: 5-8.

Hotha S, Banik RM (1997). Production of alkaline protease by Bacillus *thuringiensis* H14 in aqueous two phase systems. J. Chem. Technol. Biotechnol. 69: 5-10.

Joo H, Kumar G, Park G, Kim K, Paik S, Chang G (2002). Optimization of the production of an extracellular alkaline protease from *Bacillus horikoshii*. Process Biochem. 38: 155-159.

Kalisz HM (1988). Microbial proteinases. Adv. Biochem. Eng. Biotechnol. 36: 1-65.

Kole MM, Draper I, Gerson DF (1988). Production of protease by *Bacillus subtilis* using simultaneous control of glucose and ammonium concentration. J. Chem. Technol. Biotechnol. 41: 197-206.

Landau NS, Gornova IB, Gulikova IB, Vimik AD (1995). Immobilization of Bacillus firmus cells in cellulose tri acetate fibers and films and their use for proteinase biosynthesis. Microbiology (USSR), 66: 310-315.

Lee JK, Kim YO, Kim HK, Park SY, Oh TK (1996). Purification and characterization of a thermostable alkaline protease from *Thermoactinomyces* sp. E79 and the DNA sequence of theencoding gene. Biosci. Biotechnol. Biochem. 60: 840-846.

Lee YH, Chang HN (1990). Production of alkaline protease by Bacillus licheniformis in an aqueous two phase system. J. Ferment Bioeng. 69: 89-92.

Nehete PN, shah VD, Kothari RM (1985). Profile of alkaline protease production as a function of composition of the slant, age transfer and isolate number and physiological state of culture. Biotechnol. Lett. 7: 413-418.

Pinar-Calik P, Tomlin G, Oliver S, Ozdamarc T (2003). Overexpression of a serine alkaline protease gene in *Bacillus licheniformis* and its impact on the metabolic reaction network. Enzyme Microb.Technol. 32: 706-720.

Puri S, Beg QK, Gupta R (2002). Optimization of Alkaline Protease Production from Bacillus sp. by Response Surface Methodology. Curr. Microbiol. 44: 286-290.

Uyar F, Baysal Z (2004). Production and optimization of process parameters for alkaline protease production by a newly isolated *Bacillus* sp. under solid state fermentation. Process Biochem. 39: 1893-1898.

Varela H, Ferrari MD, Belobradjic L, Weyrauch R, Loperena ML (1996). Effect of medium composition on the production by a new Bacillus subtilis isolate of protease with promising unhairing activity. World J. Microbiol. Biotechnol 12: 643-645.