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Effective production of recombinant esterase *Bacillus brevis* using a pH-controlled fed-batch culture

Edgardo E. Tulin

Philippine Rootcrops Research and Training Center, Leyte State University, Baybay, Leyte, Philippines

ABSTRACT

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An automated two-component substrate (polypepton plus glucose) feeding strategy with a pH-stat modal fed-batch culture using a high pH limit was developed to effectively produce esterase from a protein-hyperproducing *Bacillus brevis* HPD31 harboring the plasmid pHSC131 which carries the *Bacillus stearothermophilus* esterase gene. Highest activity of the secreted esterase (34 U/ml) was obtained when the concentrations of polypepton and glucose in the nutrient feed solution were 250 g/l and 41.60 g/l, respectively. The absence and excessive amount of glucose in the nutrient feed solution were ineffective for extracellular esterase production because without glucose cell growth was minimal while excessive amount of glucose favored cell growth at the expense of esterase production. The feed rate, automatically controlled by a direct signal of pH change, at 0.30 ml/pulse was found optimum for extracellular esterase secretion. The activity of the secreted esterase was increased more than eight times from 4 U/ml in the conventional batch culture to 34 U/ml obtained in this study. The esterase productivity was likewise increased more than three-fold.

Keywords: automated substrate feeding, *Bacillus brevis*, pH-stat modal fed-batch culture, esterase

Correspondence: E. E. Tulin *Present Address:* Philippine Rootcrops Research & Training Center (PhilRootcrops),Leyte State University, Baybay, Leyte, Philippines.*Telefax:* (053)335-2616 or 3982. *E-mail:* rootcrop@philwebinc.com. *Website:* www.philwebsoft.com/philrootcrops.

INTRODUCTION

The production of proteins of commercial interest is one of the objectives in microbial technology. The genus *Bacillus* has received considerable attention because of its ability to secrete large amounts of proteins. Under optimal growth conditions, *B. brevis* strain HPD31, produced about 30g/l of protein into the medium (the extracellular protein is mostly derived from the middle wall protein of the cellwall) (Takagi *et al.*, 1989). This strain produces minimal amount of protease during cultivation so that protein secreted remains steadily in the medium even during prolonged cultivation (Udaka *et al.*, 1989). These properties make *B. brevis* HPD31 a very attractive host for the expression of foreign proteins.

Fed-batch fermentation has been acknowledged as an effective approach for the production of biological products (Yamane and Shimizu, 1984; Yamane *et al.*, 1989). The purpose of fed-batch culture is to achieve a considerable degree of freedom in the control of nutrient(s) concentration and to extend the productive period of a traditional batch process. Fed-batch production of a desired metabolite is generally characterized by the interaction between cell growth and nutrient consumption,the dependency of the cells desired metabolite biosynthesis dynamics on the concentration of the nutrient fed, and the increase in culture broth volume. In addition to the traditional microbial reaction processes, an emphasis has recently been made on the importance of nutrient feeding for both effective expression of transcription promoters and optimal secretion of heterologous proteins (Park and Ramirez, 1990).

The use of pH to control substrate feeding is one approach used in fed-batch culture and is usually applied to microbial culture systems where pH increases or decreases during cultivation (Suzuki *et al.*, 1990). Fed-batch cultures controlled by a direct signal of pH change have been employed for the production of biomass and vitamin B12 from *Pseudomonas* AM1 and *Klebseilla* (Nishio *et al.*, 1977), dextransucrase from leuconostoc mesenteroides (Brown and McAvoy, 1990) and thiostrepton from *Streptomyces laurentii* (Suzuki *et al.*, 1987). A pH-stat modal technique using the high limit has also been reported to control the growth rate of a methanol-assimilating bacterium, *Protomonas extorquens*, by regulating the feed rates of methanol plus peptone (Suzuki *et al.*, 1990).

Previously, we reported the characteristics of the batch culture of *B. brevis* HPD31 harboring a plasmid which carried the *Bacillus stearothermophilus* esterase gene (Udaka *et al.*, 1989). Carboxyl esterase (EC3.1.1.1) catalyzes the hydrolysis of carboxylic esters and has received attention because of its application in developing flavor in foodstuffs. Recent interest in the potential applications of esterase in enzyme biotransformations has generated an impetus to investigate sources and properties of microbial esterases. In microaqueos environments such as organic solvents, esterases may be used to promote esterification, intersterification, transesterification, ester synthesis, aminolysis, or peptide synthesis. Due to its expected thermostability, esterase derived from *B. stearothermophilus* is of great interest to industry and diagnostics.

Under batch conditions, esterase production of *B. brevis* HPD31 was limited to 4 U/ml. The recombinant host was grown in a complex medium consisting of glucose (G), polypepton (PP) and yeast extract (YE) and it was observed that during cultivation the pH rose steadily due to ammonium ion derived from PP after the mid-exponential phase of growth. We exploited this finding and formulated a strategy of fed-batch culture where nutrient feeding was automatically linked with the pH rise. In this study, the mode of feeding substrates (single or multi-component) to the cultivation medium and the optimum feed rates of the nutrient solution were investigated to produce the encoded foreign esterase more effectively than in the batch cultures.

MATERIALS AND METHODS

Organism and culture conditions

Bacillus brevis HPD31 harboring the plasmid pHSC131 which encodes the *B. stearothermophilus* esterase gene was used throughout the study. In each experimental run, a stock culture of this organism stored in G + PP + YE medium (medium I) containing 60% glycerol at -80 °C was thawed and plated on to a G + PP + YE agar plate containing 60μ g/ml of neomycin and 1% tributyrin. Colonies exhibiting clear zones in the tributyrin plate were inoculated into a 500-ml flask containing 100 ml of G + PP + YE medium plus neomycin (60μ g/ml) as the preculture.

The optimum pH of 6.5 and temperature of 35°C for growth and extracellular production of esterase were used. In all batch and fed-batch experiments, glucose was autoclaved separately. The polypepton used was made from pancreatic caesin (Nippon Seiyaku Co., Japan).

Batch cultures

In order to determine the appropriate time to start feeding and to understand pH variation, batch cultivation using medium I was conducted. A one -liter mini-jar fementor containing 700 ml of the medium was inoculated with 50 ml of seed culture grown in a 500-ml flask to an OD 660 of 1.8-2.0 (0.8-0.9 g dry cell/liter).

Another batch culture with medium II was carried out as a control experiment of the fed-batch cultures. In this experiment, 400 ml of medium I was mixed with 320 ml of nutrient solution (NFS) composedof 250 g/l polypepton and 41.6 g/l glucose at the start of cultivation. The composition of the NFS used gave the best results among combinations of polypepton and glucose studied in the fed-batch experiments. The same inoculum size was used as described above.

The pH of the batch cultures was kept between 6.40 and 6.60 using an automatic pH control system.

Formulation of feeding strategy

From the profile of the batch culture (see Fig. 1 in results),the substrate was fed automatically in a link with the pH rise using the high limit because a steady increase in pH was observed at the later stage of cultivation. The system used in the pH-stat modal fed-batch culture with the high pH limit and the schematic time variation of pH and nutrient solution feeding is shown in Fig. 2. In this study where the pH limit was set at 6.60, both nutrient and acid solutions were supplied to the bioreactor while the pH rose to 6.60 for a definite on time as both solutions are connected to the same pH controller. Once the pH dropped below 6.60, the supplies of both solutions stopped.The pH controller (Mituwa Biosystems, Osaka, Japan) had an automatic twin timer system for both acid and NFS feeding. Variations in pH of the cultures were monitored using a recorder (Model R-54, Rikadenki Kogyo, Tokyo, Japan). The on time to one pulse was fixed at 5 seconds. The off time was fixed at

seconds. The feed rate of the nutrient was expressed as the amount of feed solution per pulse. The feed rate of the acid solution (6 N HCl) was fixed at 0.0167 ml/pulse (1 x 10 -4 mol HCl/pulse) by adjusting the speed controlling dial of the acid feeding pump (Pump 1) (Perista-Mini SJ1215, Mitsumi, Japan) while that of the nutrient solution was varied using the speed controlling dial of the nutrient feeding pump (Pump 2) (Minipuls 2,Gilson, France).

Fed-batch cultures

The fed-batch experiments were started by initially growing the microorganism batchwise in a 1-L mini-jar fermentor containing 400 ml of medium I. A 35-ml preculture with an OD 660 of 1.8-2.0 was used in all experiments. Feeding the nutrient solution was started when nearly all of the glucose initially added was exhausted. This corresponded to 22 h of the culture time.

Single- and multisubstrate feedings

First, the effect of feedings one-, two-, three-component substrates on the extracellular esterase formation and growth of *B. brevis* HPD31 (pHSC131) was investigated. The combinations of feed solution and their concentrations (listed in Table 2) were tested. The compositions of the feed solutions were based on the maximum solubility of polypepton in water.

The concentrations of yeast extract and glucose in the feed solution were relative to polypepton, and the same proportion was used as employed in batch cultivation. Feed solution without polypepton was not tested because polypepton contains peptide, which is a precursor of esterase. Prior to feeding, neomycin was added to the nutrient solution at a concentration of 60 g/ml. In this series of experiments, the feed rate of the nutrient solution was fixed at 0.15 ml/pulse.

Polyacrylamide gel electrophoresis

The proteins in the supernatant were examined by native polyacrylamide gel electrophoresis using a 12% gel. Twelve-microliter culture supernatants taken at appropriate time intervals of cultivation were treated with one microliter of 1% β– mercaptoethanol and used for electrophoresis. Duplicates of the samples were applied on the same gel. After electrophoresis, the gel was cut in half. Esterase activity was demonstrated by incubating one of the gels for 20 minutes according to the procedure of Sundberg et al. (9) but using α -napthyl butyrate as substrate. The otherhalf of the gel was stained with Coomassie brilliant blue and destained with a solution composed of 10% methanol and 7.5 acetic acid.

RESULTS

Batch cultures

Figure 1 shows the results of cultivating *B. brevis* HPD31 (pHSC131) under batch culture using medium I. The profiles of pH variation, glucose andpolypepton consumptions, and the activity of the excreted esterase are also presented. The pH of the culture started to increase after 20 h of cultivation.At this time, glucose was nearly consumed and the concentration of ammonia increased. After 22 hours, the pH reached 6.60, and this period was considered the appropriate time to start feeding. The maximum esterase activity obtained was 4.45 U/ml afetr 24 h of cultivation.

In the control experiment of fed-batch cultures (Table 1, medium II), the activity of the excreted esterase was limited to 7.5 U/ml. This was attained at a longer cultivation time (78 h), and as a result, the esterase productivity was comparatively low (96 U/l h).

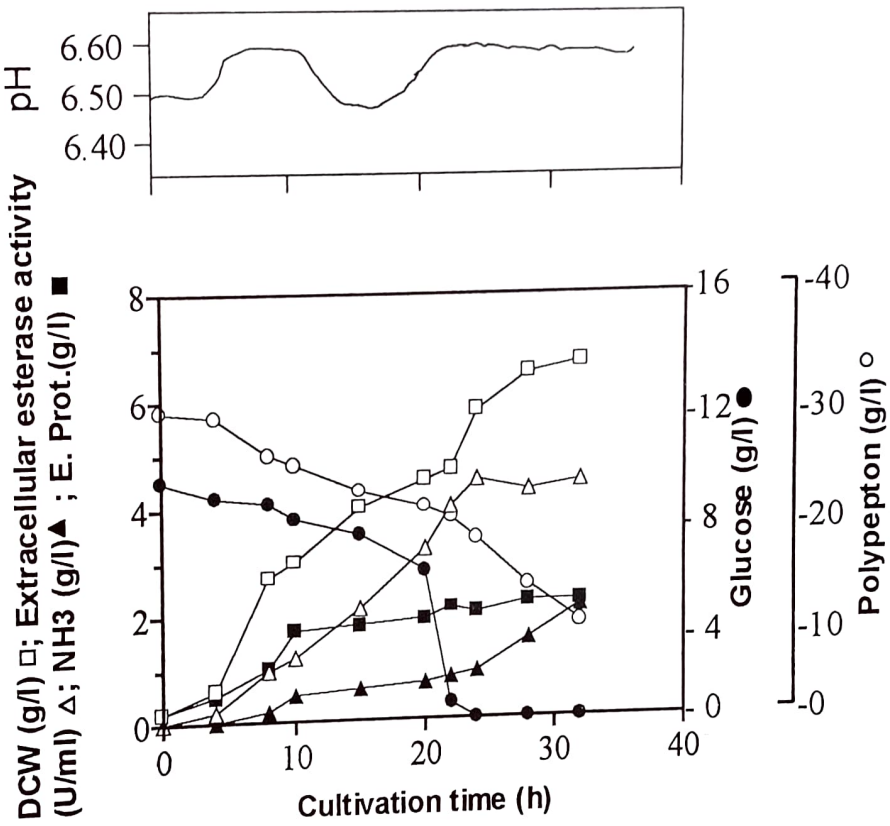

Fig. 1. Profiles of batch cultivation of B. brevis HPD31 harboring pHSC131. The composition of the medium used (medium I) is shown in Table 1.

Two-component substrate feeding

In the single and multi-substrate feeding experiments, the PP + G combination produced the best result among the four feed combinations tested. In order to further investigate the variations in the secretion of the esterase with respect to a two-component substrate feed mixture, different feed rates (ml/pulse) were tested. The pump connected to the feed solution was adjusted to give three feed rates: 0.15, 0.30,and 0.67 ml/pulse.

The concentration of polypepton in the nutrient solution was fixed at 250 g/l while different concentrations of glucose were examined: 0, 10.42, 20.83, 83.2, and 166.4.

Table 1. Results of batch cultures

	Medium I	Medium II
Glucose (g/l)	8	24.8
Yeast extract (g/l)	5	2.5
Polypepton (g/l)	30	140
Culture time (h)	32	78
μ_{max} (h -1)	0.42	0.49
$Y_{x\,glucose}$ (g/g)	0.62	0.55
$Y_{x/polypepton}$ (g/g)	0.43	0.57
Final cell concentration (g/l)	6.80	14.60
Cell productivity (g/l h)	0.21	0.18
Maximum esterase activity (U/ml)	4.45	7.50
Esterase productivity (U/l h)	139	96

The media were also supplemented with 60 g/ml neomycin as selection pressure. Culture time was set at maximum cell concentration.

μ_{max} (h -1) - maximum specific growth rate

$Y_{x/glucose}$ (g/g) - growth yield from glucose

$Y_{x/polypepton}$ (g/g) - growth yield from polypepton
Cell productivity (g/l h) - final cell concentration/culture time
Esterase productivity (U/l h) - maximum esterase activity/culture time

Analytical Procedures

Two-milliliter samples were withdrawn at regular time intervals for various analyses. The growth o f the culture was monitored by measuring the optical density at 660 nm (OD 660) using a Spectronic 20 spectrophotometer (Shimadzu, Kyoto, Japan). The OD 660 values were converted into dry cell weight, DCW (g/l), using a predetermined calibration curve between OD 660 and the DCW.

The amounts of glucose and ammonium ion in the culture broth were determined using the glucose B-test (Wako Chemicals, Osaka , Japan) and Nesslers Reagent, respectively. The amounts of extracellular protein and peptides derived from polypepton in the culture broth were measured using the method of Lowry *et al.* (1951).

The extracellular esterase activity was measured using the non-emulsion method of Yamane (16) with tributyrin as a substrate. One unit of esterase activity was defined as the amount of enzyme, which liberated 1 mol of butyric acid in one minute of reaction at 37°C.

Table 2. Results of fed-batch cultures: Effect of single- and multisubstrate feeding [a]

Polypepton (g/l)	250	250	250	250
Yeast extract (g/l)	0	41.7	0	41.7
Glucose (g/l)	0	0	66.7	66.7
Amount of nutrient solution (g) [b]	253	72	172	160
Final volume (ml) [b]	753	603	692	686
Maximum cell concentration (g DCW/1)	10.4	11.4	19.2	18.2
Maximum esterase activity (U/ml)	13.5	16.5	19.9	15.9
Culture time for maximum esterase activity (h)	90	84	90	78
Enzyme productivity (U/l h)	109	82	153	140

[a] Feed rate = 0.15 ml/pulse
Culture time was set at 90 h.
enzyme productivity (U/l h) = maximum esterase activity/culture time

Fed-batch cultures

Effect of single- and multi-substrate feedings

In this experiment, the feed rate of the NFS was maintained at 0.15 ml/pulse. The results are summarized in Table 2 and in Fig. 2. when PP or PP + YE was used as NFS, the maximum cell concentration was low and the esterase activity of the supernatant was also relatively lower. Thus, these NFS's were ineffective for enzyme production. When the three components were present in the NFS, cell growth was improved but after 70 h, cell concentration already decreased. The esterase activity was limited to only 15.9 U/ml. With PP + G, the cells continued to grow although slowly, and the esterase activity increased. The higher maximum esterase activity of 19.9 U/ml was obtained from the PP + G combination as compared to the PP + YE + G combination which only gave 15.9 U/ml. Thus, in terms of extracellular esterase production, the feed combination of PP + G gave the highest activity and productivity of the enzyme as compared to all other feed combinations tested (Table 2).

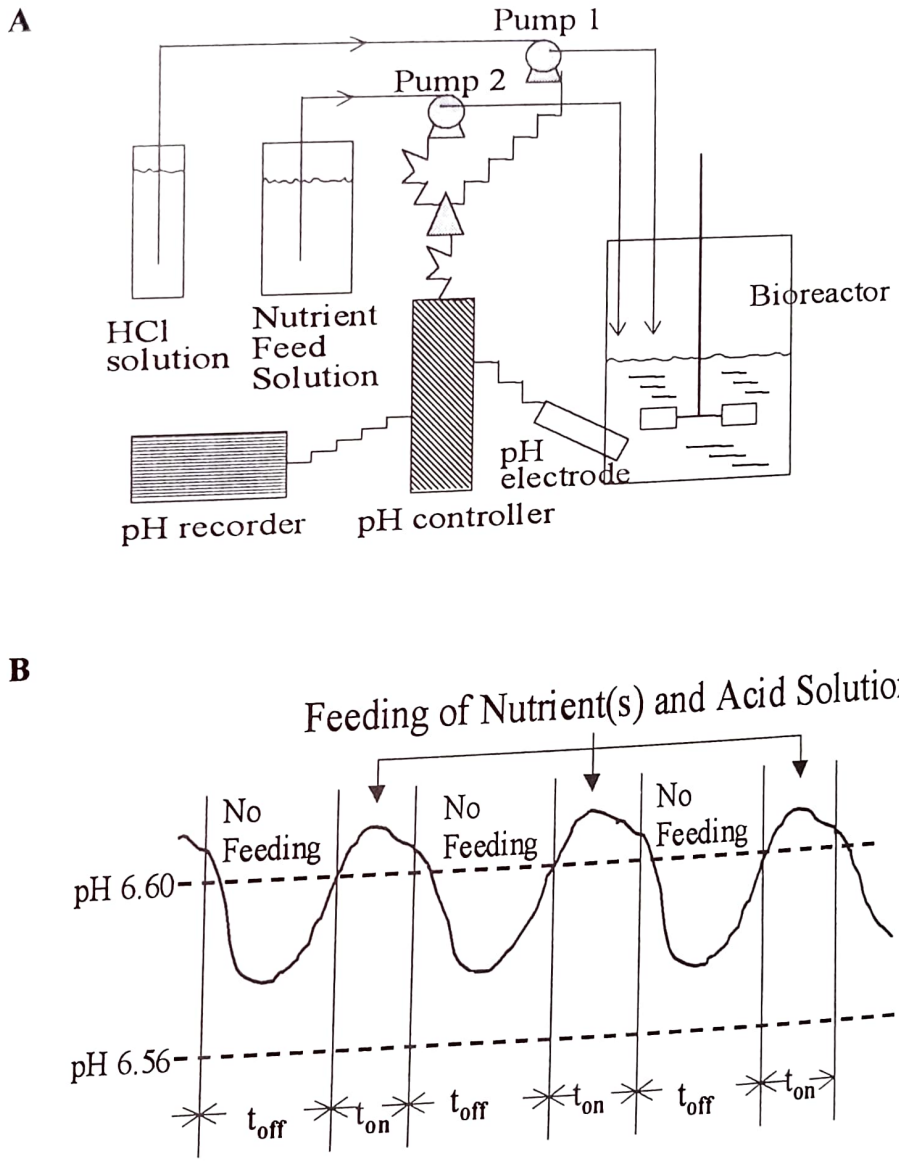

Fig. 2. Automated two-component substrate feeding in a pH-stat modal fed-batch culture. A, Bioreactor set-up; B, Schematic diagram of acid and nutrient addition using the high limit of pH 6.60. t_{on} is the on time of 5 seconds (1 pulse). t_{off} is the off time of 20 seconds.

Two-component substrate feeding

Based on the above findings, we focused our strategy on the two-component substrate feeding strategy. Using a feed rate of 0.15 ml/pulse, the activity of the secreted esterase did not vary significantly when the glucose level was set at 0, 10.4, 20.8 and 66.7 g/l (data not shown). Thus, higher feed rates of 0.30 and 0.67 ml/pulse were tested. Using a feed rate of 0.30 ml/pulse, a higher concentration (166.4 g/l) of glucose in the NFS supported good cell growth (Table 3). However, esterase was not always high. The highest extracellular esterase activity was obtained when the concentration of glucose in the NFS was 41.6 g/l. At this glucose concentration, the final cell concentration was about 20 g/l and the esterase activity reached 34 U/ml.

As a basis of comparison, the total culture volume of the fermentor was set at 750 ml, which corresponded to about 320 of NFS fed. As shown in Table 3, increasing the concentration of glucose in the NFS from 0 to 166.4 g/l at a feed rate of 0.30 ml/pulse increased the culture time from 62 to 144 h.

When the feeding rate was further increased to 0.67 ml/pulse, the culture time ranged from 72 to 86 h, but the extracellular esterase activity remained low. Comparison between the three feed rates using the same compositions of NFS showed that the feed rate of 0.30 ml/pulse gave the best results. The activity of 34 U/ml was obtained, which was eight times as much as that of the conventional batch culture. In terms of esterase productivtiy, NFS containing 250 g/l polypepton and 41.6 g/l glucose at a feed rate of 0.30 ml/pulse gave the best result (298 U/ h), which was more than three times than that obtained under the batch cultivation (96 U/l h) (Table 1).

Activity of the secreted esterase

The activity of the secreted esterase which was demonstrated using α-napthyl butyrate as substrate showed a prominent band in the correct position (Fig 4), which was absent in both the wild type, and the recombinant strain harboring the expression vector only.

Table 3. Results of automated two-component substrate feeding by pH-stat modal fed-batch culture [a]

Feed rate (ml/pulse)	0.30	0.30	0.30	0.30	0.30	0.30	0.67	0.67
Polypepton (g/l)	250	250	250	250	250	250	250	250
Glucose (g/l)	0	10.4	20.8	41.6	83.2	166.4	0	41.6
Culture time (h)	62	68	108	114	114	114	72	86
					(690)[b]	(665)[b]		
Final cell concentration (g DCW/l)	14.6	16.5	16.8	20.4	18.6	20.4	13.8	17.0
Final total cell mass (g DCW)	10.9	12.4	12.6	15.3	13.9	15.3	10.3	12.7
Final esterase activity (U/ml)	18	20	27	34	22	17	14	18
Esterase activity (U/l h)	290	294	250	298	177	132	198	209

[a] Time when total volume in the fermentor reached 750 ml, except in the marked (b) cases.
Final total cell mass (g DCW) = final cell concentration (g DCW/l) x final volume (l)
Esterase productivity (U/l h) = final esterase activity (U/l)/ culture time (h)

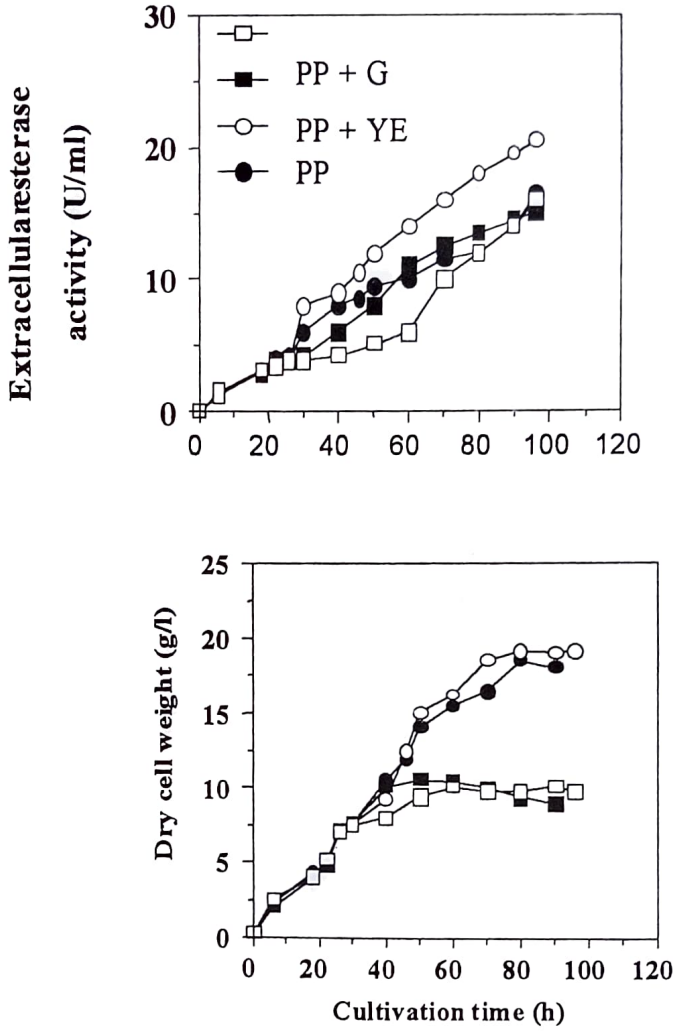

Fig. 3. Effect of single- and multicomponent nutrient feed solution on the growth and esterase activity of *B. brevis* HPD31 harboring pHSC131 in a pH-stat modal fed-batch culture. The arrow indicates the starting time of feeding. The compositions of the nutrient feed solution are listed in Table 2.

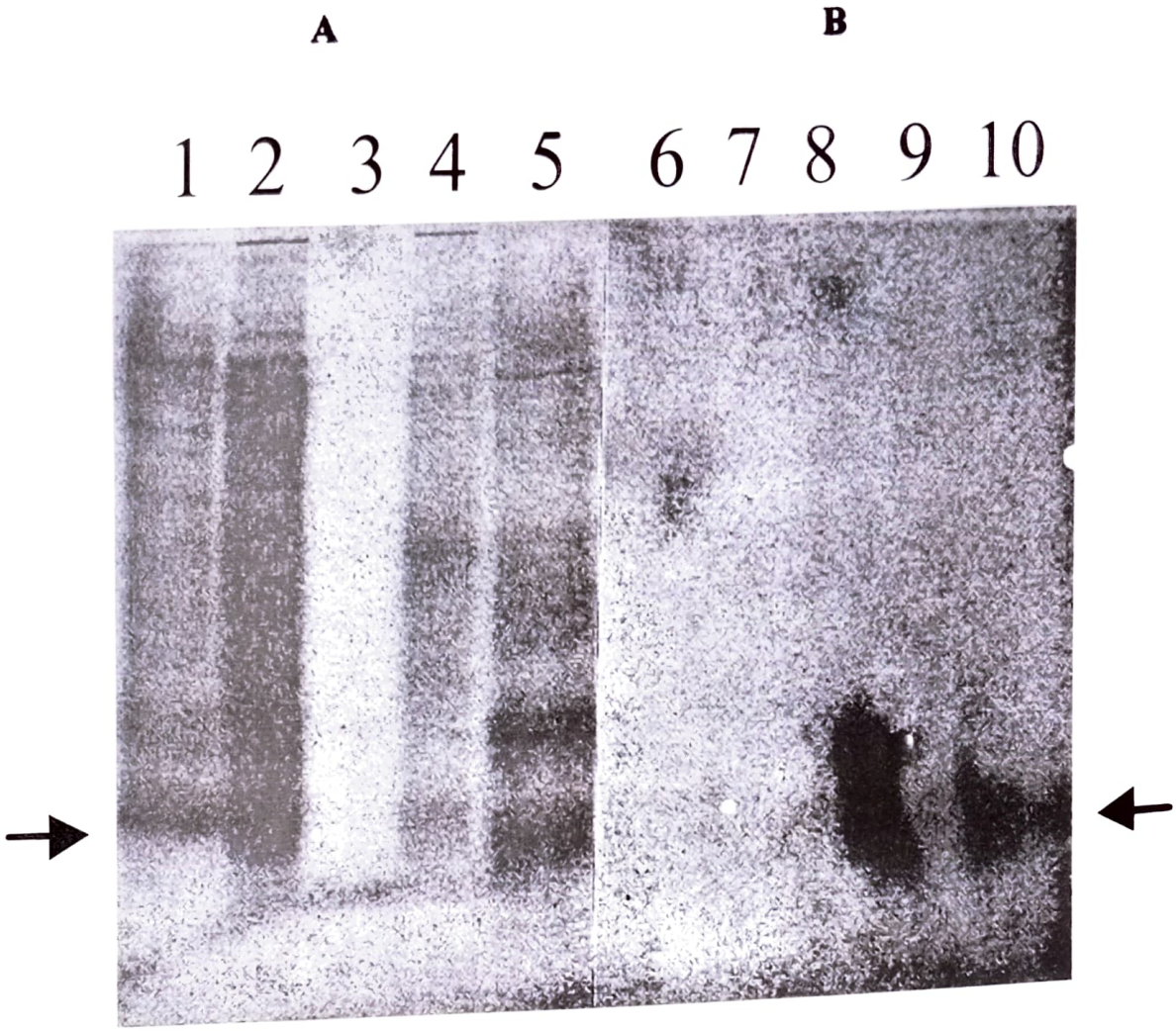

Fig. 4. Native gel electrophoresis of culture supernatants from *B. brevis* HPD31. (A) Coomassie brilliant blue staining; (B) Esterase activity staining. Lanes 1 and 10, *B. brevis* HPD31/pHSC131 after 36 h of cultivation; lanes 2 and 9, *B. brevis* HPD31/pHSC131 after 72 h of cultivation; lanes 3 and 8, *B. brevis* HPD31/pHSC131 medium plus inoculum at 0 h; lanes 4 and 7, *B. brevis* HPD31/pNH300 (expression vector) after 72 h of cultivation; lanes 5 and 6, *B. brevis* HPD31 wild type after 72 h of fed-batch cultivation.

DISCUSSION

In this paper, we present an effective approach of producing recombinant esterase from a protein-hypersecreting *Bacillus brevis* strain HPD31 using a pH-stat modal fed-batch culture. The pH-controlled fed-batch culture using the high pH limit was applicable in this regard because at the later stage of cultivation, the pH of the culture medium steadily increased. The supply of substrate to the culture medium was controlled by the rise in pH and this was effective in regulating the growth of *B. brevis* HPD31 because the mechanism of enzyme secretion was found to be growth-dependent. A higher bacterial cell growth was unfavorable for enzyme secretion thus, a balance between cell growth and enzyme secretion was necessary to effectively secrete the enzyme into the medium.

Changing the concentration of glucose in the nutrient fed was found to be effective in regulating cell growth and esterase secretion. It was necessary to incorporate optimum amount of glucose in the feed solution because without it, cell growth was compromised and the activity of the secreted enzyme remained low. High concentration of antibiotic thiostrepton was produced from *S. laurentii* when the glucose concentration was kept lower than 5 g/l (Suzuki et al, 1987). Addition of excessive amounts of glucose in the cultivation medium was ineffective for extracellular enzyme production. Earlier reports showed repressive effects of glucose on secondary metabolite formation such as benzylpenicillin (Fishman and Biryukov, 1974 ; Soltero and Johnson, 1953) and also of protease (Moon *and Parulekar, 1991*). In the case of polypepton, the presence of relatively high concentration of polypepton in the culture medium during the course of extracellular enzyme synthesis may result in the corresponding increase in the size of the RNA precursor pool causing an increase in the rate of transcription. This effect was suggested by Coleman *et al* (1975) who studied the extracellular enzyme synthesis in *B. amyloliquefaciens*.

The strategy used in this experiment employing a pH-stat modal fed-batch culture using a high pH limit depends on the production of ammonia in the culture of the medium. Using polypepton alone as feed solution i.e., in the absence of glucose, a continued increase in the production of ammonia occurred. As a result,the pH continued to increase and feeding was relatively faster. This condition was not favorable for enzyme secretion.

When glucose was added with polypepton in the feed solution, it took some time before the pH increased again because the cells had to utilize first the glucose added. In this case, feeding of the nutrient solution was comparatively slower resulting in a regulated bacterial cell growth and effective esterase secretion. In addition to the optimum concentration of glucose in the NFS, effective esterase secretion also depended on the substrate feed rate. Using a feed rate of 0.15 ml/pulse, esterase secretion remained low even if the highest possible glucose concentration (166.4 g/l) was added. A higher feed rate of 0.67 ml/pulse was also ineffective in producing the recombinant protein. The most effective feed rate was obtained at 0.30 ml/pulse.

Finally, the effectiveness of the pH-stat modal fed-batch culture using a high pH limit used in this study was confirmed by conducting a control experiment whereby the initial medium (400 ml consisting of 8 g/l yeast extract, and 30 g polypepton) and the total amount of NFS (320 ml consisting of the best PP + G combination of 250 g/l polypepton and 41.6 g/l glucose) was mixed together at the start of batch culture (Table 1, medium II). The activity of the secreted esterase was 7.45 U/ml as compared to 34 U/ml obtained in the best fed-batch system.

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