Enhanced Production of Alkaline Protease by *Teredinobacter turnirae* in Fed-Batch Fermentation using a Concentrated Medium

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To increase cell concentration and the production of alkaline protease, fed-batch cultures with standard medium (1x) or concentrated medium (2x or 5x) as feed were performed. In a fed-batch culture with standard medium as feed, the biomass concentration reached 9.6 g L^{-1} and the protease activity obtained was 8500 U mL^{-1} compared to 3000 U mL^{-1} obtained by growing *Teredinobacter turnirae* cells with standard PB-medium in batch culture. For further increase of process efficiency, a high density fed-batch process was developed, where concentrated medium is fed to the cells. Fed-batch process using concentrated medium was evaluated for its ability to improve cell growth and protease titer of *T. turnirae*. It was found to result in up to 3.9-fold increase in final protease activity compared to batch culture control.

Key words:
Teredinobacter turnirae, alkaline protease, fed-batch culture, concentrated medium

Introduction

A major objective of fermentation in research and industry is to maximize the volumetric productivity (g L^{-1} h^{-1}), i.e. to obtain the highest possible amount of product in a given volume within a certain time. Therefore, it is important to develop a cultivation method that allows production of the desired product to a high concentration with high productivity and yield. High cell densities are a prerequisite for high productivity. Therefore, fed-batch culture has been widely employed for the production of various bioproducts including primary and secondary metabolites, proteins, and other biopolymers. Fed-batch is generally superior to batch processing and is especially beneficial when changing nutrient concentrations affect the productivity and yield of the desired product.[1,2] The application of optimization techniques to fed-batch operations in order to maximize productivity were previously recorded.[3-5]

By extending the culture period, the fed-batch operation enables a higher final cell density and productivity than batch operation. Recently, concentrated medium formulations have been proposed as feed to further enhance the efficiency of fed-batch processes. Strategies for fed-batch process development could, in general, be classified into two categories: the "bottom-up" approach and the "top-down" approach. The bottom-up approach for fed-batch protocol development consists of supplementation of the culture with medium components that are identified as being quickly consumed or depleted. In the simplest case, the nutrient feeds consist of a single nutrient or a combination of carbon and an energy source. In general, the bottom-up approach requires the long-term investment of manpower and time. Identification of the important nutrients and determination of the optimal composition of nutrient cocktails to be used for feeding require much analytical work. The bottom-up approach is also very cell line specific: a high-yield fed-batch protocol developed for one cell line or clone has to be redesigned or re-optimized in order to perform optimally with another cell line or clone. For instance, this approach was applied for the production of alkaline protease by feeding sucrose and/or NH_4Cl.[6] Who found that, fed-batch culture by feeding both sucrose and ammonium chloride was superior to batch fermentation of *T. turnirae* in producing alkaline protease. The maximum protease production rate of P = 158 U mL^{-1} h^{-1} was nearly 2.6-fold greater than values observed in batch operations. On the other hand, the "top-down" approach for fed-batch protocol development consists of feeding the culture with complete medium in order to quickly achieve cell growth and product titer improvements with no need to

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identify limiting medium components and formulate customized nutrient cocktails. Complete medium feeding can be accomplished easily by the use of medium concentrates. This technique has been shown to increase cell densities and final product titers dramatically.[7-10] Therefore; fed-batch fermentation experiments were carried out using concentrated media to study its effect on cell growth and protease production by *T. turnirae* cells.

Proteases are among the most valuable commercial enzymes. Most industrial uses of proteases are in food processing, pharmaceuticals, peptide synthesis, meat tenderization, medical diagnosis, baking, brewing,[11] deproteinization of shrimp and crab-shell waste[12] and dehairing of hides and skins.[13,14] Due to its increased economic importance, research is being carried out throughout the world to isolate hyperactive strains for the production of protease and to optimize various parameters for maximizing its production.[15] Who studied the optimization of protease production in a bubble column bioreactor operated in a fed-batch mode, using a newly isolated *Pseudomonas* sp. RAJR 044. It was evident from the optimization results that the fed-batch fermentation favored increased protease production in a reduced reaction time while sustaining the growth rate, characteristic of this particular strain. Another interesting research about the enhancement of alkaline protease production by *Bacillus sphaericus* using fed-batch culture was recently published.[16] It was found that, by using suitable feeding strategies, the protease activity and its productivity in a fed-batch process was increased by 44 % over that in the batch process, primarily due to the longer maintenance of increased rates of growth and enzyme production by providing continuous and controlled supply of additional substrate and nutrients.

During a search for novel protease, *T. turnirae* was iosolated from the gland of Deshayes of the marine shipworm, *Psiloteredo healdi*.[17] The industrial potential of alkaline protease produced by *T. turnirae* and its unique properties were described elsewhere.[18-20] Therefore, and based on the improvement in protease production by *T. turnirae* using a simple fed-batch culture of carbon and/or nitrogen source published elsewhere,[6] the current work presents the evaluation of 1-, 2-, and 5x concentrated medium for *T. turnirae* producing a novel alkaline protease and demonstrates the general applicability of this approach for successful short-term fed-batch process development. The effects of 1-, 2-, and 5x medium addition on cell growth, protease production and nutrient consumption are discussed.

Materials and methods

Microorganism and cultivation conditions

Bacterial culture of *T. turnirae* was generously supplied by Prof. Dr. Moreira (UMBC, Baltimore, USA). The basal medium (Placket-Burman PB) used throughout all experiments was prepared as previously described.[18] It contains in (g L^{-1}): KCl 0.4; $MgSO_4 \cdot 7H_2O$ 1.9; $MgCl_2 \cdot 6H_2O$ 1.5; $CaCl_2 \cdot 2H_2O$ 0.4; HEPES 4.9; Antifoam 289 (φ = 0.1 %); sucrose 5.0; NH_4Cl 1.0; solution A 10 mL; trace metal solution 1 mL. Solution A consisted of g L^{-1}: $K_2HPO_4 \cdot 3H_2O$ 24; Na_2CO_3 12; $Fe_2(SO_4)_3$ 0.3. The trace metal solution consisted of g L^{-1}: H_3BO_3 2.9; $MnCl_2 \cdot 4H_2O$ 21.6; $ZnSO_4 \cdot 2H_2O$ 0.2; $Na_2MoO_4 \cdot 2H_2O$ 0.04; $CoSO_4 \cdot 7H_2O$ 0.05 and $CuSO_4 \cdot 5H_2O$ 0.08. Pre-inocula were developed by transferring freshly grown cells from LB-agar plate into 250-mL Erlenmeyer flasks, each containing 50 mL of PB-medium and incubating the flasks at 30 °C and n = 120 min^{-1} on a rotary shaker for 24 h to serve as inoculum for the fermenter.

Fermentation conditions for fed-batch culture

All fed-batch fermentations were carried out in a baffled 3 L stirred tank bioreactor Bioflow III (New Brunswick Scientific Co., New Brunswick, NJ, USA) with an initial volume of 1.5 L. Temperature was controlled at 30 °C. Mixing was carried out with three 6-bladed flat-blade impellers operating at n = 250 min^{-1}. The air flow rate was maintained at 1 L L^{-1} g^{-1}, based on the initial culture volume. The initial pH of the medium was adjusted to 7.0 and was not controlled during the course of fermentation but the decrease in pH during cultivation was not significant. Foam control was achieved by addition of silicone antifoaming agent (Fluka, Switzerland). Oxygen partial pressure was measured with a polarographic oxygen probe (Ingold, Germany). Initially the reactor was filled with 1.5 L of standard PB-medium. During fed-batch mode 1 L of standard medium or concentrated medium was fed using a peristaltic pump with a flow rate of Q = 65 mL h^{-1} for 15 h and then switched back to batch fermentation until the end of cultivation. The starting time of the feeding pump and the feed flow rate were determined based on the previously obtained data.[6]

Analytical procedures

All determinations reported in this work were performed in triplicate and experiments were executed at least in duplicate; the results are given as the mean values.

Biomass determination

Biomass was determined spectrophotometrically (OD 600 nm) and converted to grams of cell dry mass (g CDM) by comparison with a standard conversion curve prepared for *T. turnirae* cells.

Enzyme assay

Volumetric proteolytic activity was determined using w = 0.8 % azocasein as substrate as previously described.[21]

Ammonium chloride determination

The ammonium ion concentration was determined using ammonia assay kit (AA-0100, Sigma Chemical Co., St. Louis, MO, USA). The ammonia readings were converted to NH_4Cl concentrations.

Sucrose concentration

Sucrose was determined enzymatically by using a sucrose determination kit (SCA-20, Sigma Chemical Co., St. Louis, MO, USA).

Results and discussion

Feeding of substrate and nutrients were employed to overcome their inhibition and a set of experiments, were carried out by feeding concentrated medium in order to optimize feed medium composition. 1.5 L of medium was used for initial batch culture and feeding (at a constant flow rate of Q = 65 mL h^{-1}) was started after 24 h of fermenter inoculation using a pre-calibrated peristaltic pump for about 15 h.

Fed-batch culture with standard medium as feed

A first approach of a fed-batch culture with standard medium as feed was tested. Experimental time course of the culture is shown in Fig. 1A. The vertical solid lines denote the time of starting and termination of feeding. The culture was started as a abatch with cell dry mass of 0.348 g L^{-1} after inoculation. Cells grew immediately and reached a biomass concentration of 3.3 g L^{-1} after 24 h. The protease produced achieved an activity of 3100 U mL^{-1}, which is comparable with the enzyme activity produced in a classical batch-fermentation using PB-medium as a culture medium.[6] Then, the fed-batch using 1 L of 1x PB-medium was started as previously described in Materials and Methods. This leads to a significant improvement in both cell growth and protease yield. Up to this point, cells grew significantly faster than in batch culture and reached a cell dry mass concentration of 9.6 g L^{-1} after 48 h cultivation, which kept more or less constant until the end of the fermentation process. On the other hand, production of protease in culture medium was also improved in the same manner as cell growth. The maximal protease activity achieved was 8500 U mL^{-1}. This indicates that using the proposed fed-batch strategy increased protease production 2.7-fold in comparison with classical batch cultivation.

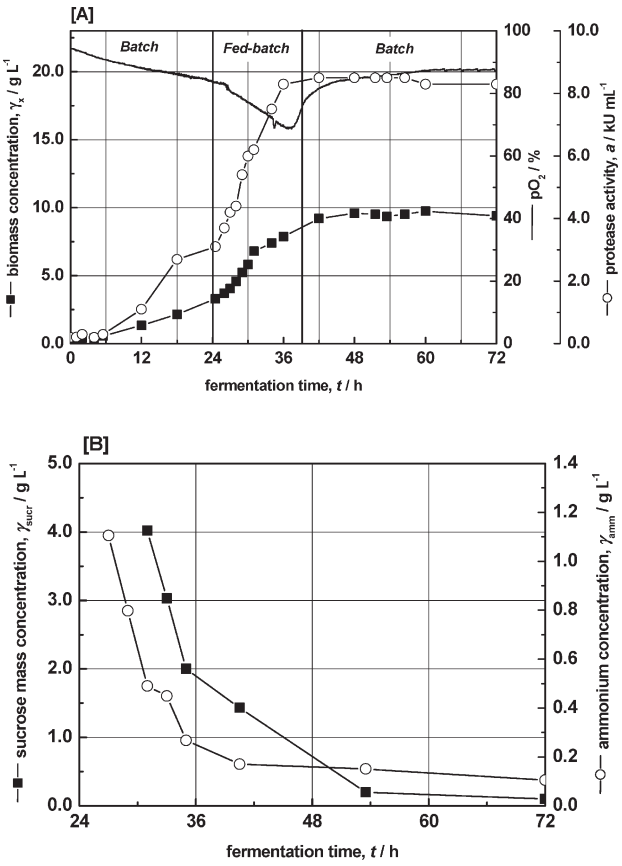


Fig. 1 – *Fed-batch fermentation with standard medium as feed. A: Cell mass concentration γ_x, protease activity and dissolved oxygen profile. B: sucrose mass concentration γ_{sucr}, NH_4Cl mass concentration γ_{amm} vs. culture time in stirred tank bioreactor.*

In addition, sucrose and NH_4Cl consumption were monitored during the fed-batch and the last batch fermentation period, as shown in Fig. 1B. The obtained results revealed that both sucrose and NH_4Cl added during the fed-batch process were consumed. A final mass concentration of sucrose and ammonium chloride were 0.2 and 0.151 g L^{-1} after a cultivation time of 72 h, respectively. Therefore, concentrated medium will be used as feed in the following experiments in order to investigate any possible further improvement in both cell growth and enzyme production.

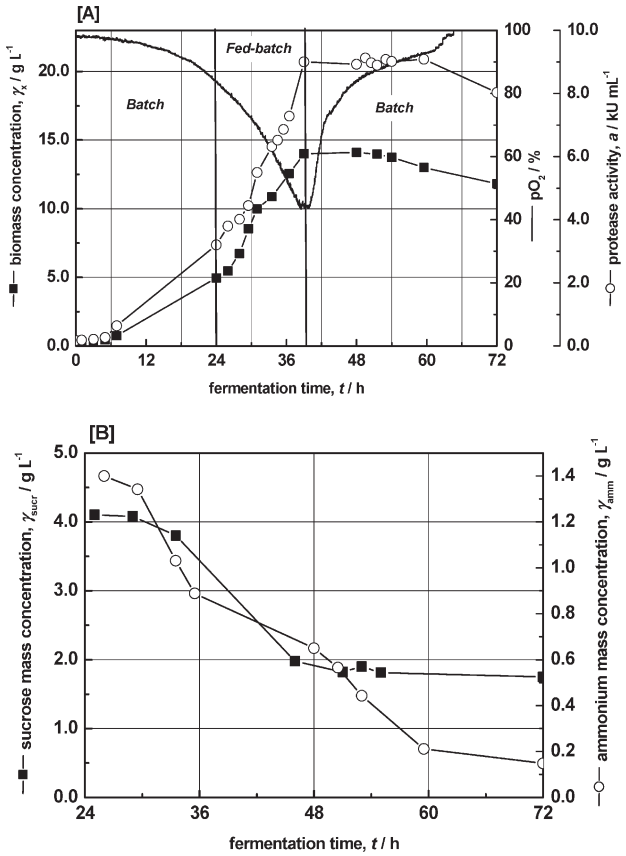
Fed-batch cultivation with concentrated medium

In an effort to improve cell growth and protease titers of *T. turnirae* cells, fed-batch with 2 or 5x

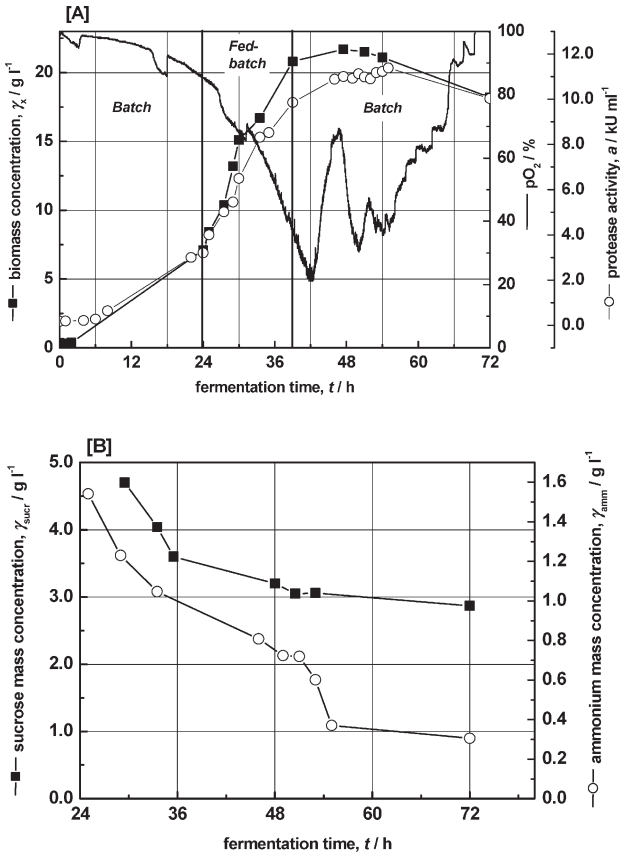
concentrated medium was evaluated. The profiles of cell growth, protease activity, sucrose consumption and other parameters for fed-batch with different concentrated medium are presented in Fig. 2 and 3. When concentrated medium was fed to the broth during the cultivation, the maximum cell concentration and protease activity were higher than those obtained with feeding of standard medium.

Maximum cell growth of 21.7 g L^{-1} was obtained at 48 h whereas protease activity attained its maximum of 11 382 U mL^{-1} after 55 h in case of feeding 5x concentrated medium. In all cases, culture performance was improved compared to batch culture. An increase in final protease activity on order of 367 % was achieved, showing that complete medium concentrates are a very useful tool for achieving high protease production. In addition, a closer look at the profiles of various parameters showed that the fall in DO, sucrose and ammonium chloride were much more pronounced in fed-batch process with concentrated medium when compared with feeding of complete standard medium, due to higher cell growth and utilization of sucrose and nutrients.

The profiles of cell growth and product concentration and their rates of formation indicated that higher rates of growth ($d\gamma_x/dt$) could be maintained for a longer period by feeding concentrated media in fed-batch stage, thereby producing higher cell concentrations. In additions, the continuous availability of substrate and nutrients could result in the maintenance of higher rate of enzyme production over a longer period and hence, higher enzyme activity in fed-batch culture. Other authors also obtained improvement in process yield using concentrated medium as feed during fed-batch cultivation. For instance, 10-fold concentrated medium was used to increase cell concentration of *Haematococcus pluvialis* and the accumulation of astaxanthin production.[9] Moreover, pulses of the concentrated 5x modified LB medium were added in a controlled manner to increase the production of streptokinase.[8] The biomass concentration reached 1.8 mg mL^{-1} dry cells and the activity obtained in the supernatant was 180 plasmin units mL^{-1} compared to 17 plasmin units mL^{-1} obtained by growing *E. coli* cells in simple LB medium.



F i g . 2 – *Fed-batch fermentation with concentrated medium (2x) as feed. A: Cell mass concentration γ_x, protease activity and dissolved oxygen profile. B: sucrose mass concentration γ_{sucr}, NH_4Cl mass concentration γ_{amm} vs. culture time in stirred tank bioreactor.*



F i g . 3 – *Fed-batch fermentation with concentrated medium (5x) as feed. A: Cell mass concentration γ_x, protease activity and dissolved oxygen profile. B: sucrose mass concentration γ_{sucr}, NH_4Cl mass concentration γ_{amm} vs. culture time in stirred tank bioreactor.*

Comparison between batch and fed-batch cultivation

The results of the fed-batch culture with standard medium and with concentrated medium as feed were compared with batch culture. The efficiency of the fed-batch culture with concentrated medium as feed was significantly higher compared to the other culture methods. As compared with batch culture, fed-batch culture produced enhanced cell growth and protease activity (Table 1 and Fig. 4). In fed-batch with standard medium and concentrated medium, the maximal protease productivity showed an impressive increase from 92 (batch culture) to 253 U mL^{-1} h^{-1} using fed-batch with 5x medium.

Our obtained results with feeding increased sucrose and all nutrients are in agreement with the previously published results for pullulan production by strain of *Aureobasidium pullulans* using batch and fed-batch culture.[22] They found that, for fed-batch culture, the highest values of pullulan mass concentration (24.5 g L^{-1}) and pullulan productivity (3.5 g L^{-1} d^{-1}) were obtained in the culture grown with feeding substrate mass concentration 50 g L^{-1} sucrose and all nutrients. Shin *et al*. reported that the inhibitory effect of high sugar concentration could be overcome.[23] Using a step-wise feeding mode, resulted in an exopolysaccharide mass concentration of 58 g L^{-1} upon exhaustion of the sugars in the fed-medium, a total 10 % sucrose concentration was introduced in the fermentation vessel in three stages intermittently.

Table 1 – *Cell growth and protease production for batch and various fed-batch fermentations*

Fermentation type	γ_x g L^{-1}	P_{max} U mL^{-1}	Maximal productivity P/U mL^{-1} h^{-1}
Batch cultivation[18]	3.1 (at 50 h)	2890 (at 50 h)	92
Fed-batch with standard medium (1x)	9.6 (at 48 h)	8500 (at 42 h)	231
Fed-batch with concentrated medium (2x)	14.1 (at 48 h)	9000 (at 39 h)	231
Fed-batch with concentrated medium (5x)	21.7 (at 48 h)	11382 (at 55 h)	253

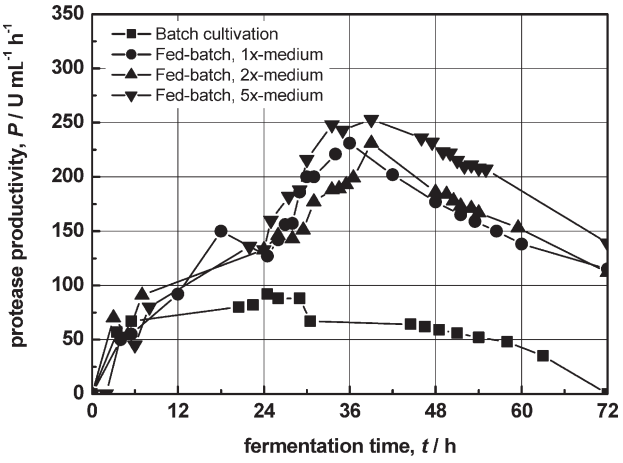


Fig. 4 – *Comparison between batch and fed-batch cultivations based on protease productivity in stirred tank bioreactor*

High-density fed-batch culture is a widely adopted methodology to enhance process productivity in microbial fermentations. Experiments to investigate the effects of medium feeding on cell growth and anthocyanin production by *Perilla frutescens* cells were studied.[24,25] Cultivations at initial sucrose mass concentrations of 15, 20 and 30 g L^{-1} without feeding were taken as controls. The intermittent feeding of all medium components was conducted at different cultivation time. The results indicate that cell growth and anthocyanin accumulation are favored by a higher initial sucrose mass concentration. Furthermore, the cell mass was increased using feed that contained higher sucrose concentration. Wang *et al*.[26] claimed that the operation of a cell culture at a low initial sucrose mass concentration (20 g L^{-1}) in combination with sucrose feeding at a later stage could effectively improve cell growth and both the production and productivity of taxane.[26]

T. turnirae is able to utilize several organic and inorganic sources of nitrogen.[27] Ammonia is preferred since it is assimilated easily and supports high growth rates.[28] Our obtained results with increasing ammonium chloride concentration and all nutrients in serial fed-batch fermentations could be compared with the effect of ammonium chloride on plasmid DNA production in high cell density batch culture.[29] Using of 2 g L^{-1} of ammonia resulted in dry biomass concentrations of 20 g L^{-1} and a maximum product mass concentration of 50 mg L^{-1}. The volumetric productivity also reached its maximum value under these conditions.

Conclusions

Batch cultivation using very high concentrations of carbon and nitrogen sources could inhibit both cell growth and product yield. Therefore, the fed-batch mode, as a semi-continuous cultivation strategy, is generally favored by too many microorganisms to reach high cell density and enhance the production yield. Classical fed-batch fermentations have been carried out with *T. turnirae* using sucrose

and ammonium chloride as feeding solution, which leads to an increase in protease titer of 2.6-fold higher than that obtained with batch culture.[6] Another fed-batch approach was applied in this work, to study a possible improvement in cell growth and protease activity using concentrated medium. Two different concentrated media (2x and 5x) were evaluated here for their ability to improve cell growth and protease activity. Increases in final protease titers on the order of 294 – 394 % were achieved, showing that complete medium concentrates are a very useful tool for reaching high protease production.

In conclusion, with the addition of concentrated medium (5x) as a feed, it was possible to obtain high cell density and protease production. This helped to enhance the protease activity 3.9-fold as compared to the activity achieved in the batch culture.

ACKNOWLEDGEMENTS

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List of symbols

A – activity, U mL^{-1}
n – stirring speed, min^{-1}
P – productivity, U mL^{-1} h^{-1}
P_{max} – maximal protease (activity) production, U mL^{-1}
Q – volume flow rate, mL h^{-1}
w – mass fraction, %
γ – mass concentration, g L^{-1}
γ_x – cell dry mass concentration, g L^{-1}
$d\gamma_x/dt$ – cell growth rate, g L^{-1} h^{-1}
φ – volume fraction
CDM – cell dry mass

Subscripts

amm – ammonim chloride, (NH_4Cl)
sucr – sucrose
max – maximum

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