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High production of alkaline protease by *Bacillus licheniformis* in a fed-batch fermentation using a synthetic medium

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SUMMARY

High production (9016 U/ml) of alkaline protease by *Bacillus licheniformis* has been achieved. A 49% increase in production was achieved by the method used as compared with a batch process. By using a synthetic medium and a fed-batch operation controlled by the Advanced Fermentation Software (AFS) package, it was found that the keys to high production of protease are: (i) to maintain a low concentration of glucose (< 0.43 g/l) in the medium; (ii) to control pH at a certain level (pH 6.50) in the culture; and (iii) to use rough type colonies as the starting culture. Our fed-batch fermentation process successfully simulates and surpasses ordinary batch fermentation processes. By using ammonium sulfate instead of soy bean flour as the only nitrogen source, an expected benefit was the elimination of unpleasant odors caused by natural organic nitrogenous components in the media. This would improve the industrial production environment.

INTRODUCTION

Bacillus licheniformis is capable of producing a variety of products, such as bacitracin, penicillinase, alkaline phosphatase, ribonuclease, α-amylase and protease [5,13]. These products are widely used in such industries as applied chemistry, tannery, food and feed, etc.

Natural media have been used for a long period of time in bacterial studies on protease fermentation using bacterial producers. Although high production has been obtained and media are inexpensive, drawbacks of the media are obvious. The relationships among medium composition, cell growth and protease formation are unclear, owing to the complicated compositions of media and the mixing of cell and solid ingredients. Therefore, it is difficult to compare the various studies of these relationships [10]. Moreover, natural organic nitrogenous components in the media produce unpleasant odors during the fermentation.

Since the 1960s, many important results have been obtained from a series of studies concerning the metabolic regulation and the kinetics pattern of protease produced by species of *Bacillus* under conditions of chemostat cultures and synthetic or semi-synthetic media. Glucose was used as the only carbon source. In addition to inorganic ammonium, additives (yeast extract, sodium glutamate, etc.) were also used as nitrogen sources [2–6,12,13]. It is generally acknowledged that there is little or no production of protease during the exponential growth phase in batch culture [4].

There were two aims in this study, to get a high production of alkaline protease by *Bacillus licheniformis*, and to eliminate unpleasant odors during the fermentation. The first has been achieved in fed-batch fermentation using our own synthetic medium. Preliminary studies on the relationship between the fermentation conditions and protease production have been performed. A simple inorganic compound, ammonium sulfate, instead of soy bean flour was used as the only nitrogen source in the medium. An expected benefit was that the unpleasant odors disappeared. This would contribute to an improvement in the industrial production environment.

MATERIAL AND METHODS

Seed culture

Bacillus licheniformis PW-109, a commercial production strain, was used in this investigation. This strain was grown on agar slants composed of 10 g/l beef extract, 10 g/l peptone and 5 g/l sodium chloride at 36 °C for 24 h. The seed was developed by transferring the cells grown on the slant into 250-ml flasks, each containing 100 ml me-

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dium. Seed flasks were cultivated on a New Brunswick Scientific Co. G-25 Gyrotory Incubator Shaker at 36 °C and 200 rpm for 7 h.

Fermentation

A 5-l working volume BioFlo III fermentor (New Brunswick Scientific Co., Inc.) was used in this investigation. This fermentor is equipped with an internal multiloop controller which regulates temperature, agitation, dissolved oxygen, pH and nutrient addition. The AFS (Advanced Fermentation Software) of NBS was used to control glucose addition. This software was executed on an IBM model PS/2 30 personal computer. Basic fermentation medium was composed of 6 g/l glucose, 10 g/l $(NH_4)_2SO_4$, 8 g/l Na_2HPO_4, 4 g/l KH_2PO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.02 g/l $CaCl_2$ and fortified with trace amounts of Cu^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+} and growth factors. The seed medium was identical to this formula. SAG-471 silicone antifoam (300 ppm) was added before sterilization. Ammonium hydroxide (7.5 N) was used both to regulate pH and as a supplementary nitrogen source. An NBS MX-3 Biosampler was used to take samples automatically.

Alkaline protease estimation

The samples were centrifuged (Damon/IEC HN-S centrifuge) at 2500 rpm for 20 min. The supernatant was diluted 100- or 200-fold. One ml of the diluted sample was preheated in a 40 °C water bath. One ml of 2% casein solution (prepared with a pH 11 borax buffer solution) was added into the sample. Reaction temperature was 40 °C. After precisely 10 min, 2 ml of 0.4 M trichloroacetic acid was added into the sample to terminate the reaction. A blank correction was necessary for each sample. After centrifugation, 1 ml of the reactant supernatant was taken and mixed with 5 ml of 0.4 M sodium carbonate and 1 ml of 0.1 N Folin-Ciocalteu's phenol reagent. The sample was incubated at 40 °C for 20 min to develop color and the optical density was measured at 680 nm (Perkin-Elmer Lambda 4B). The slope of the standard curve determined with L-tyrosine was used in the calculation of protease activity. One unit of protease activity is defined as the amount that liberates 1 μg L-tyrosine per min under the testing conditions described above.

Cell concentration

The sample was diluted 10- or 40-fold with de-ionized water. The supernatant of the sample as the blank was similarly diluted. The optical density values of both at 600 nm vs. water were measured in the same spectrophotometer. Cell concentration was calculated as the difference of OD values between the sample and the blank.

Glucose concentration

Glucose concentrations were determined with a commercial enzyme assay kit (Sigma).

RESULTS AND DISCUSSION

Effects of initial pH, pH set point and initial concentration of glucose on protease production

Some operating conditions and the results for six experiments are tabulated in Table 1. Unless otherwise mentioned, the following operating conditions were held constant for a series of fermentation experiments. After inoculation, the volume of the medium was about 4 l in each experiment. The volume of added glucose was about 200 ml. Regardless of what the initial concentration of glucose was, the total amount of glucose used in each run was 50 g/l. Ammonium hydroxide was used to regulate pH and the total volumes added were 90–135 ml in those experiments. Experiments were run at 36 °C, 0.5 v/v per

TABLE 1

Some operating conditions and results for six runs

Run no.	Initial pH	Set point of pH	Initial glucose concentration (g/l)	Glucose addition start time (h)	Carbon/ nitrogen ratio	Peak turbidity of cells (OD)[a]	Peak activity time (h)	Protease activity (U/ml)
1	6.78	6.50	20	27	4.27	2.25	44	8096
2	6.49	6.50	16	23	3.64	2.40	44	8296
3	6.53	6.50	6	18	3.60	2.39	36	9016
4	6.94	7.20	16	16	4.42	2.20	34	2120
5	7.00	7.00	16	13	3.90	2.38	32	2920
6	6.84	7.00	6	17	3.56	2.41	40	4168

[a] 10-fold dilution.

min air flow rate and 400 rpm initial agitation speed, which was also the minimum speed for dissolved oxygen (DO) control. The initial DO level was calibrated to 100% and the set point of the DO control was 30%. If the DO level dropped below this point, the agitation speed would increase automatically to maintain the DO level until a maximum speed of 950 rpm was reached. When initial glucose was depleted, the pH value increased. The pH trend was a useful measure of glucose depletion. The glucose addition was actuated by this trend through the user-defined algorithms of AFS. An additional flow rate in the vicinity of 0.21 ml/min corresponding to the flow rate reading of 9% for the nutrient pump control was used to maintain the low concentrations of glucose in the medium.

The six runs can be divided into two groups according to their peak values of protease activity. High values were observed in runs No. 1, 2 and 3 and lower values in runs No. 4, 5 and 6. The major variations in operating conditions between these two groups were the initial pH values and the set points of the pH controller. The initial pH values in the high production group were lower than those in the lower production group. The pH set points were 6.50 in runs No. 1, 2, and 3; 7.20 in run No. 4 and 7.00 in runs No. 5 and 6. It is evident that pH plays a major role in protease production. The optimal pH set point was 6.50 in our experimental investigation.

Runs No. 1, 2 and 3 belong to the high production group, but different values of protease activity were obtained because of variations in the initial concentration of glucose. It was found in our studies that protease production was inhibited by glucose. Frankena et al. [4] reported that in glucose-limited chemostat cultures the specific rate of protease production was maximal at a specific growth rate of 0.22. They asserted that exocellular protease was subjected to glucose repression above this specific growth rate. For the same reasons, among runs No. 1, 2 and 3, the highest production was obtained from the batch whose initial concentration of glucose was the lowest. In the other two batches, which contained a high initial concentration of glucose, most of the initial glucose made little or no contribution to protease production.

The high initial concentration of glucose caused other disadvantages. In runs No. 1, 2 and 3, the quality of seeds was nearly identical, but higher concentrations of glucose resulted in a longer lag phase, and inevitably a longer fermentation time. In runs No. 4 and 5, although initial concentrations of glucose were also high, the shorter lag phase was attained by using a larger amount of inoculum.

It was observed that under high initial concentrations of glucose, a large amount of viscous material formed in cultures. Bergey's Manual of Systematic Bacteriology states that levan is produced extracellularly from sucrose and raffinose by *Bacillus licheniformis* [11]. The viscous material in our studies is also inferred to be a certain kind of polysaccharide. Its actual properties are as yet unclear.

It could be seen that when the initial concentration of glucose was 2 g/l, the protease production would eventually be sufficient, as long as the viscous material which formed in the earlier part of the run could be used after glucose depletion, such as in run No. 1. However, in most of such high initial glucose cases, in addition to runs No. 1–6, especially in runs for which the pH was maintained at 7.00, the viscous material could not be used. Once the glucose was added, excess viscous material formed, rendering those fermentations unusable. *Bacillus polymyxa* has been reported [7] to form a high-viscosity agar-like polysaccharide at the optimal pH of 7.00–7.50. In some runs, if the initial pH was 7.00 and the pH was maintained at 7.00, although the initial concentration of glucose was only 16 g/l, a large amount of the viscous material formed, rendering the fermentation unusable. It can be considered that the pH plays another major role in the formation of the viscous material.

Effects of colony type, cell mass and carbon/nitrogen ratio on protease production

In the course of our studies, two morphological types of colonies were seen on the slants. The rough type was the high production strain, while the smooth type was the low production strain. Similar results have been reported previously [1,4,8,9]. Unstable results were not expected in our studies, thus more attention was paid to seed slants. As a short-term measure, the criteria for the production strain used in the factory was implemented. Isolation of high production colonies was performed often and the number of transfers of the strain from slant to slant was conducted as little as possible.

Table 1 shows that the variations of the peak turbidity of the cultures were not large from batch to batch. Although the OD value in run No. 6 was close to the values in runs No. 2 and 3, the protease activity in this batch was only about half of those high activities. However, in order to obtain high protease activity, a certain amount of cell mass is necessary. Nevertheless, just as the experiment revealed, if fermentation conditions were not appropriate, protease production was still poor, even though the amount of cell mass was large.

In this work, the total amount of glucose was fixed, the amount of ammonium sulfate was also fixed and ammonium hydroxide as a supplemental nitrogen source was automatically added for pH control. The average carbon/nitrogen ratios (g/g) of both high and lower production groups, of 3.83 and 3.96, respectively, were very close. However, the carbon/nitrogen ratio in run No. 3 could be adopted for higher production.

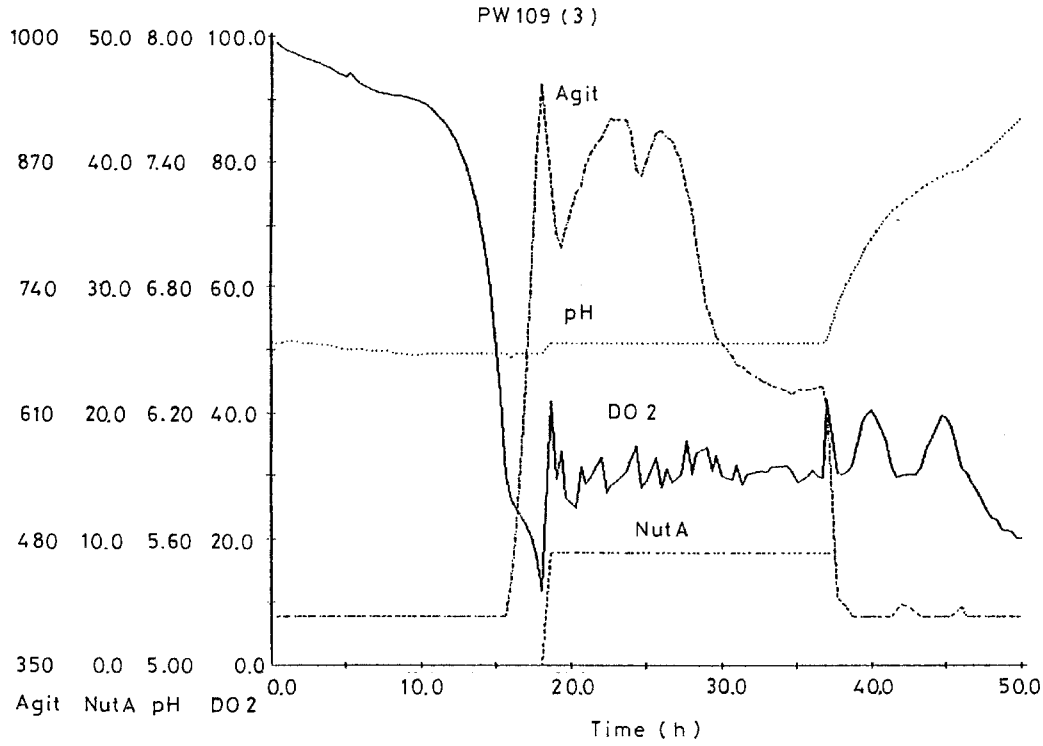

Fig. 1. Profiles of the DO level, pH value, glucose addition and agitation speed for run No. 3.

Time profile of the highest production run

The profiles of the DO level, pH value, glucose addition and agitation speed for run No. 3 are shown in Fig. 1. This is an original drawing plotted by a printer running on the AFS software. Profiles of the cell growth (OD values by 40-fold dilution), glucose consumption and protease activity for the same run are shown in Fig. 2. As can be seen, the DO level and glucose concentration gradually decreased with the slow growth of cells. A 12-h period of lag phase was observed. The cell growth and glucose consumption increased substantially during 12 to 16 h. This time can be looked upon as the pre-exponential growth phase. The depleted concentration value of glucose, which was only 0.38 g/l, was observed at 18 h. Glucose addition started at this moment due to an increase of the pH caused by the depletion of glucose. The glucose concentrations were always less than 0.43 g/l during the glucose addition period (18 to 37 h), except that the concentrations were a little bit higher during the first several hours. The exponential growth phase with a doubling time of 2.2 h occurred during 16 to 26 h. The high requirement of oxygen by cells during this exponential growth phase was indicated by the phenomenon that the DO mostly remained at low levels, between 25–35%, and the agitation promptly increased and then remained at high speeds, between 785–950 rpm. When the reduction of cell growth resulted from the glucose limitation, a decrease of oxygen requirement occurred during 26 to 30 h in the culture. Thus it was observed that, although the DO still remained near the set point level, the agitation speed dropped markedly. This phenomenon indicates the occurrence of another period

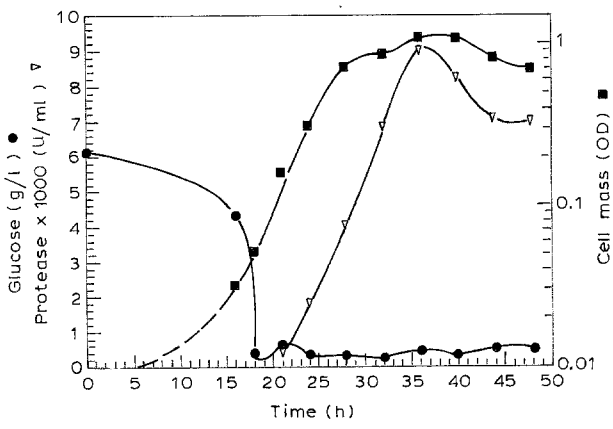

Fig. 2. Profiles of the cell growth, glucose consumption and protease activity for run No. 3.

considered as the post-exponential growth phase. The termination of glucose addition occurred at 37 h. The stationary phase occurred during 30 to 45 h.

The pH was automatically regulated by ammonium hydroxide, so that the medium pH remained at the set points, which were 6.48 before and 6.52 after starting to add glucose, respectively. This tiny change of the set point was for ease of formulating user-defined equations, when AFS was used to actuate the nutrient pump. In the later stage of this run there was evidence of autolysis. The termination of glucose addition speeded up this process. The switch of the energy source, presumably to amino acids, and cell autolysis caused a marked increase of the pH.

From the profile of protease activity in Fig. 2, it can be observed that there was little or no formation of protease during the earlier exponential growth phase. The activity of protease was only 416 U/ml at 21 h. A rapid increase of protease activity began at the middle stage of the exponential growth phase. The protease production lagged behind the growth of cells by about 8 h. The peak activity of protease was found to be 9016 U/ml at 36 h. Then protease activity gradually decreased with cell autolysis.

It was felt that the formation of spores might be correlated with the production of protease. Through microscopic observation, a small number of spores was found during the later part of the exponential growth phase, and a large number of spores was found during the stationary phase in run No. 3. The same phenomenon was also observed in runs No. 1 and 2. Low protease production along with a small number of spores existed during the stationary phase of runs No. 4, 5 and 6. However, we cannot affirm that a large number of spores must correspond to high protease production. Bulthuis et al. [1] reported that the smooth type of *B. licheniformis* (not protease producing) can sporulate also.

Protease activity of 6064 U/ml was obtained in the batch culture using the same strain at 37 h under the same conditions of temperature, aeration, initial agitation speed and set point of the DO control. The medium was composed of 40 g/l starch, 30 g/l soy bean flour, 4 g/l Na_2HPO_4 and 1 g/l yeast extract. The initial pH was 8.47 and pH control was not used in this batch fermentation as is done in the real industrial process. The pH profiles in both batch and fed-batch cases were very different. The reasons are understandable. The media corresponding to both cases have substantial differences. It has already been noted that the protease production is repressed by glucose. If glucose is used as the only carbon source in the medium, glucose feeding is necessary. Starch is always used as the carbon source in batch fermentation processes which are non-feeding. These processes are still the established ways of industrial protease production in China. Our fed-batch process successfully simulated and remarkably surpassed the ordinary batch type fermentation processes. Also, the unpleasant odors were completely eliminated.

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