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Effects of Culture Conditions on Protease Production by *Streptomyces clavuligerus* Growing on Soy Bean Flour Medium

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ABSTRACT

The influence of nitrogen and phosphate sources on the production of extracellular protease activity by *Streptomyces clavuligerus* has been investigated. The experiments were carried out in batch fermentation using soy-bean flour as nitrogen source and potassium phosphate dibasic as phosphate source. High protease yield was obtained after 24 h of fermentation with an initial pH of 7.0. The maximal protease activity (112.68 and 88.72 U/mg) was obtained the phosphate concentration of the 21 and 29 m*M* for strains 3585 and 644, respectively. With regard to the nitrogen concentration in both strains, the maximal protease activity was achieved with 0.5% (154.89 U/mg and 228.36 U/mg for 3585 and 644 strains, respectively). Enzyme production appeared to be modulated by an inducer system where ammonia, complex nitrogen, and phosphate sources might have been involved.

Index Entries: *Streptomyces clavuligerus*; proteases; nitrogen source.

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INTRODUCTION

Proteases are among the most important groups of industrial enzymes; their major application is in food, pharmaceutical, and detergent industries (*1*). These enzymes account for at least 25% of total enzyme sales, with two-thirds of proteases produced commercially being of microbial origin (*2*).

Bacterial protease from *Bacillus* genus has long been used in detergents, but preparation requires cost-intensive filtration methodologies to obtain a microbe-free enzyme preparation (*1*). The protease of *Streptomyces*, however, offers an advantage, in that the mycelium can be easily removed by a simple filtration process.

A substantial amount of information has been accumulated on nitrogen and phosphate control of secondary metabolism *S. clavuligerus* (*3*), and nitrogen catabolic enzymes (*4–5*). This species is also known to produce extracellular protease activity. A partial characterization of its activity indicated that it was because of a single metallo-protease with an apparent molecular mass of 41.700 Da (*6*). However, the amount of protease produced varied greatly with the strains and the media used.

In order to obtain higher and commercially viable yields of protease, we carried out studies on protease production using soybean flour as the nitrogen source, a low-priced and easily available medium. We also examined the influence of phosphate concentration on protease production by *S. clavuligerus*.

MATERIALS AND METHODS

Microorganisms

S. clavuligerus strain NRRL 3585 and mutant 644 (isolated in medium with high concentration of clavulanic acid, 600 mg/mL), were used in this study. The microorganisms were maintained at 28°C on ISP-2 agar slants (*7*), made up of malt extract (1.0%), yeast extract (0.4%), and agar (2.0%).

Production Media and Culture Conditions

The inoculation was carried out using Erlenmeyer flasks (250 mL) containing 50 mL of the fermentation medium (MF) described by Aharonowitz and Demain (*8*), containing: glycerol (1.0%), yeast extract (0.1%), $MgSo_4 \cdot 7H_2O$ (0.06%), NH_4Cl (0.1%), K_2HPO_4 (0.435%), and 0.1 mL mineral solution ($FeSO_4 \cdot 7H_2O$ 100 mg, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot H_2O$, distilled water [100 mL], with pH initial 7.0). Culture was grown for 48 h at 28°C, with orbital shaking (200 rpm).

Growth curve experiments were carried out using Erlenmeyer flasks (500 mL) containing 125 mL of the culture medium with orbital shaking (200 rpm) over 96 h at 28°C. Samples were removed at time intervals and supernatants were used to measure protease activity and protein concentration.

The effect of a nitrogen source and its concentration on protease activity was investigated using MF medium, where L-asparagine and yeast extract were replaced by soybean flour (MSB) in the following concentrations: 0.5; 1.0; 2.0; 4.0% (w/v).

The influence of a phosphate concentration source was determined using MSB medium, with the following values: 21.0, 25.0, 29.0, 32.0 and 50.0 m*M*. All experiments were carried out in the same conditions mentioned above.

Biomass Determination

The biomass concentration was determined as mycelial dry weight after centrifugation of 10 mL samples of culture broth (all measurements were carried out in duplicate). The samples were dried at 105°C overnight until constant weight.

Analytical Methods

Protease Assay

Total extracellular protease was assayed at 25°C as described by Ginther (*9*) in culture medium previously clarified by centrifugation (12,000 × g, 5 min). Azocasein 1.0% (w/v) (Sigma, St. Louis, MO), in 0.2*M* Tris-HCl, pH 7.2, containing 1.0 m*M* $CaCl_2$, was used as substrate. One unit of activity is defined as the amount of enzyme that produces an increase in the optical density of 1.0 in 1 h at 440 nm.

Protein was determined using the method described by Bradford (*10*), with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Effect of Nitrogen Concentration on the Protease Production in Batch Fermentation

Synthesis and secretion of protease are induced by peptides or other proteinaceous substrates, such as soybean flour. Depending on the nature and level of peptides, these may induce or repress protease synthesis and secretion. The effect of different substrate concentrations on protease yield during the growth is shown in Fig. 1 A, B. The best yield was obtained by fermentation using 0.5% (w/v) of soybean for both strains. The preliminary studies showed that the optimum period for protease production was 96 h (end of exponential and beginning of stationary phase) of the fermentation for both strains used. These results are in accord with Bascarán et al., which showed that synthesis of protease starts in the post-exponential phase of growth (*6*) in *S. clavuligerus*. Kole et al. (*12*) described the same behavior in *Bacillus subtilis*. Decrease in protease activity with increase of the concentration of the nitrogen source, together with

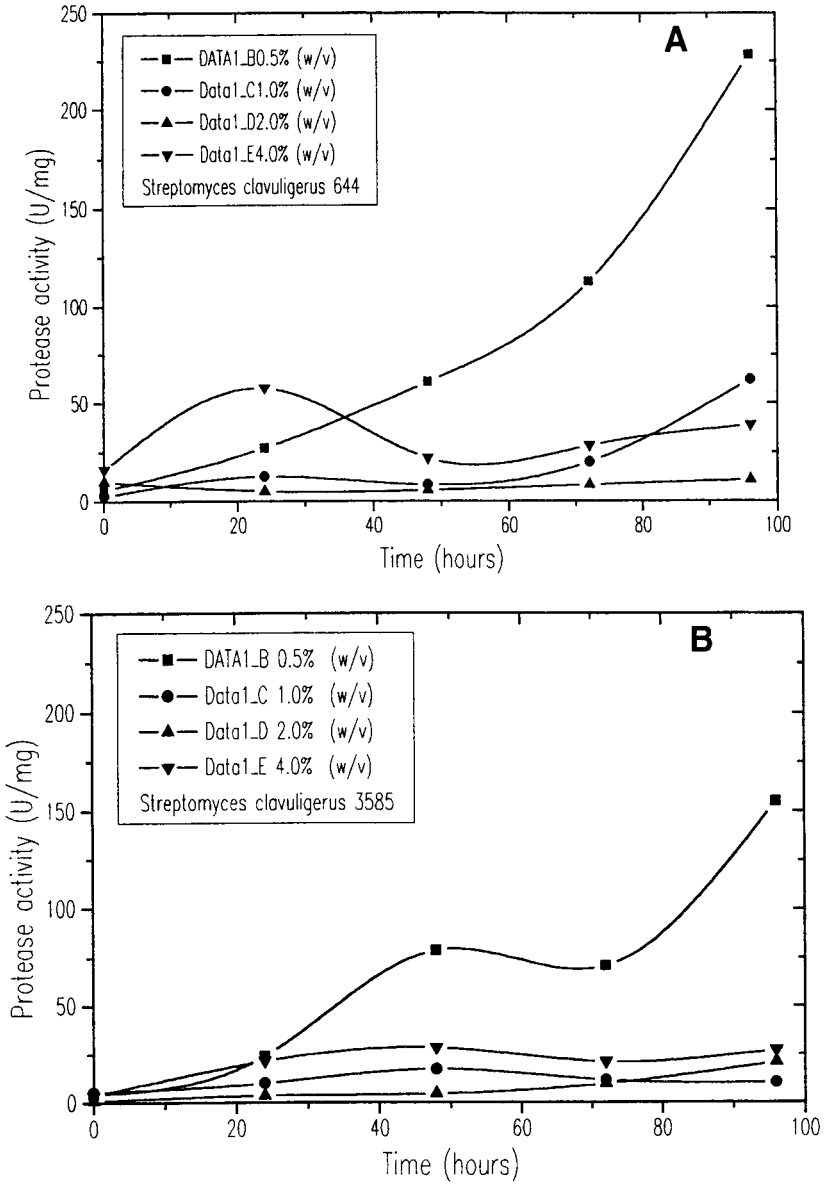

Fig. 1. Effect of nitrogen concentration on protease production by *S. clavuligerus* 3585 (**A**) and 644 mutant (**B**). Both strains were grown on soybean flour nitrogen source.

higher ammonia concentrations, also has been observed in other microorganisms, such as *Aeromonas hydrophila* (*11*) and *B. subtilis* (*12–13*). In order to explain this behavior, Bascarán et al. (*6*) has described a regulatory circuit that mediates ammonium repression of several nitrogen catabolic enzymes, but it is still not clear if this repression is by alteration of glutamine synthetase activity, which can be affected by high concentrations of ammonium, like that found in yeast such as *S. cerevisiae* (*15*), or if protease synthesis is directly modulated by this nitrogen control system (*6*).

Soybean flour is a rich source of amino acid, vitamins, carbohydrates, lipids, phosphorus, and proteins. Usually, free amino acids are the end products of proteolytic degradation of complex proteins. These amino acids present in the soybean flour can work as inhibitors for protease synthesis. At the level employed, an increase in soybean flour concentration appears to have resulted in accumulation of amino acids in *S. clavuligerus* culture. This excess can cause repression in the protease synthesis.

Experiments reported by Bascaran et al. (*6*), using *S. clavuligerus* for protease production after nutritional shift-down, indicated that initiation of protease formation is observed with decreased nutrients available. Good enzyme production was obtained using nitrogen-free medium or in presence of poorly utilized amino acids, but decreased with amino acids supporting higher growth rate.

The mechanisms by which control of protease production is achieved in many prokaryotes systems are not yet known (*1*).

Effect of Inorganic Phosphate Concentration

Phosphate plays a vital role as an effector of a large number of enzymatic reactions of primary metabolism, including synthesis of DNA, RNA and proteins, carbohydrates metabolism, cellular respiration, and control of ATP level (*1*). The overall results of batch experiments (uncontrolled pH), with different phosphate concentrations in the medium, are presented in Fig. 2 A, B; both strains showed similar behavior of pH and maximum cell mass with respect to initial phosphate concentration in the medium. In the 3585 strain, the maximum protease activity was achieved using 21 m*M* of phosphate (112.68 U/mg). A concentration up to 29 m*M* stimulate the cell growth, but inhibited protease production (Fig. 3). The increase in glucose utilization, cell growth, and production of enzymes in wild-type and recombinant microorganisms has been reported elsewhere, using an excess of inorganic phosphate in the medium, up to certain levels (*2*). However, the maximum cell mass was drastically repressed using phosphate over 29 m*M*. The pH values were below those with low phosphate concentration, but protease activity went up toward the same pH value found using 21 m*M* of phosphate (Fig. 3).

In the 644 strain, applying phosphate concentrations from 21 to 29 m*M*, the protease activity increased to a maximum of 88.72 U/mg, but over 29 m*M* its activity was strongly inhibited, showing more sensitivity to phosphate than the 3585 strain. The maximum cell mass behavior was repressed from 21 m*M*, but it was practically stable using phosphate from 25 to 50 m*M* (Fig. 2). Its final pH showed a behavior opposite to the maximum cell concentration in experiments with the pH being uncontrolled in batch culture. The decline in the pH with 21 m*M* of phosphate can be explained by the production of acidic products by cells with a high growth rate, represented by a high maximum cell mass.

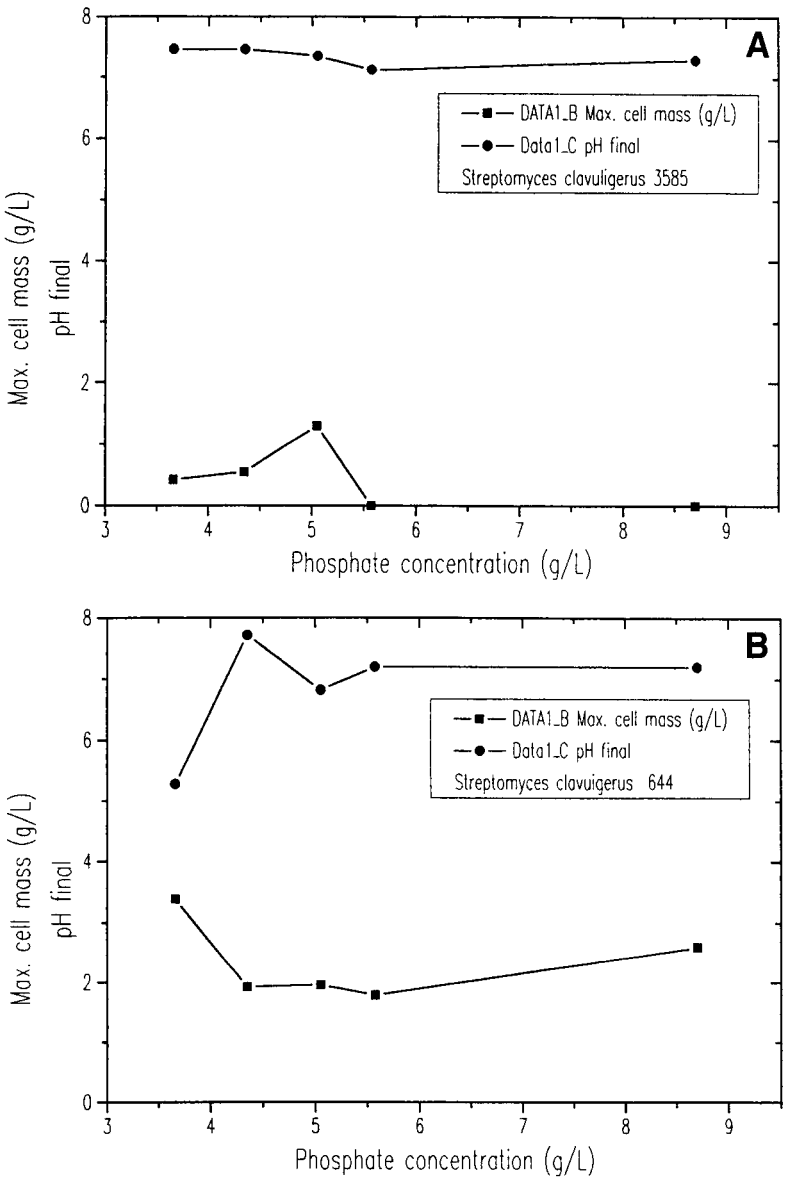

Fig. 2. Curves of maximum biomass (■) and pH (●) at different concentrations of phosphate by *S. clavuligerus* 3585 (**A**) and 644 mutant (**B**).

It has been postulated that the stimulatory effect of phosphate on exoenzyme production occurs at the level of protein translation or secretion (*14*). Therefore, phosphate may increase stability of messenger RNA, specific for protease, by inhibiting RNase activity, or it may effect the cytoplasmatic membrane so that membrane-bound ribosomes are better adapted for exoprotein translation (*2*).

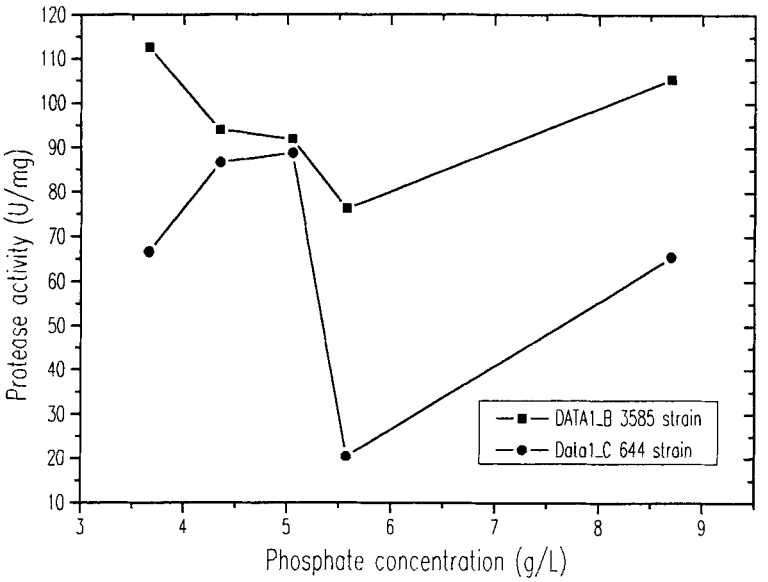

Fig. 3. Influence of different phosphate concentrations on protease activity by *S. clavuligerus* 3585 (■) and 644 mutant (●).

Production of protease seems to be very sensitive to variations in the initial phosphate concentration in both strains of *S. clavuligerus* (Fig. 3). A concentration of 21 m*M* phosphate is the best concentration for the 3585 strain and 29 m*M* phosphate is the best concentration for the 644 strain, using soybean flour as nitrogen source.

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