PRODUCTION OF EXTRACELLULAR PROTEASES BY *MUCOR CIRCINELLOIDES* USING D-GLUCOSE AS CARBON SOURCE / SUBSTRATE

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ABSTRACT

Recently, some Mucorales species have been reported as protease producers. The production of extracellular proteases by *Mucor circinelloides* using glucose as substrate was studied. Experiments were carried out with different D-glucose concentrations (40, 60 and 80 g/L). Biomass, pH and protease activity were determined. Although biomass production had reached best yields for the medium containing D-glucose in a concentration of 80 g/L, the enzymatic production was higher when the substrate concentration was reduced to 40 g/L. The yield factor for product on cell growth and the yield factor for product on carbon substrate were higher when the microorganism grew in medium containing 40 g/L glucose. The kinetics parameters suggest that this strain seems to be promising as an alternative microorganism for protease production.

Key words: extracellular proteases, D-glucose, substrate, *Mucor circinelloides.*

INTRODUCTION

Microorganisms produce a large variety of enzymes, most of which are made in only small amounts and are involved in cellular processes. Extracellular enzymes are usually capable of digesting insoluble nutrient materials such as cellulose, protein and starch, and the digested products are transported into the cell where they are used as nutrients for growth (12,18). Some extracellular enzymes are used in the food, dairy, pharmaceutical, and textile industries and are produced in large amounts by microbial synthesis (1,2).

In the fungi of the order Mucorales, members of the genus *Mucor* occur typically as saprophytes in soil, and among them, *M. miehei*, *M. pusillus* and *M. bacilliformis* have been utilized in food industry and fermented beverage (4,10,20).

Proteases are one of the most important groups of industrial enzymes and account for nearly 60% of the total enzyme sale (4,7). The major uses of free proteases occur in dry cleaning, detergents, meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds (17). Although bacterial proteases have long been used in industry, the main drawback in their use is that they require cost-intensive filtration methodologies to obtain a microbe-free enzyme preparation. On the other hand, the proteases of fungal origin offer an advantage in that the mycelium can be easily removed by filtration (19).

Several reports describe the efficient protease biosynthesis by fungi belonging to the genera *Aspergillus* (8), *Penicillium* (5), *Rhizopus* (9), *Humicola* (1), etc. Two closely related species of Zygomycete fungus, *Mucor pusillus* and *Mucor miehei*, secrete aspartate proteases, also known as mucor rennins, into the medium. The enzymes possess high milk-clotting activity and low proteolytic activity, enabling them to be used as substitutes for calf chymosin in the cheese industry (15,21).

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Investigations for protease production by many fungal cultures have shown that the producing amount of proteases varies greatly with the media used and regulatory effects exerted by the carbon sources have been described (18). Many experiments have been carried out with microorganisms cultivated with D-glucose as a substrate but there is little information about how the concentration and consumption of substrate affect enzyme production in Mucorales (7).

Few papers have been published about the variables that control the production of protease from *Mucor* (7,15). It is not known how cell concentration and substrate consumption affect enzyme production. The rate of substrate consumption and its relationship to the previous variables are fundamental to determine the kinetics of the fermentation. Kinetic equations are basic for future bioreactor design and optimization.

The aim of this work was to investigate the protease production by *M. circinelloides* using D-glucose as substrate. To do so, process kinetic parameters were evaluated against different substrate concentrations. The relation between production and growth was analyzed to determine whether the enzyme is growth-associated, non-growth-associated or a mixture of growth associated and non-growth associated. The possibility of the enzyme being affected by feedback repression was also studied.

MATERIALS AND METHODS

Microorganisms

M. circinelloides IFM 40507 was obtained from the culture collection of the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Japan. The cultures were maintained on potato dextrose agar (Difco) slants at 4ºC. Every two months the cultures were transferred to new slants to keep them viable.

Inoculum preparation

M. circinelloides spores were harvested from cultures grown for 5-7 d in Petri dishes containing Difco's potato dextrose agar at 25ºC. A stock suspension was prepared and adjusted to 1.0 x 10^7 spores/mL, using a haemocytometer for counting. The solution was then stored at 4ºC.

Fungal culture and growth conditions

The cultures were grown in a synthetic medium previously described by Hesseltine and Anderson (14) which contains 40 g/L D-glucose, 2 g/L asparagine, 0.005 mg/L thiamine, 0.5 g/L potassium phosphate and 0.25 g/L magnesium sulfate (pH 5.2). The concentration of D-glucose in media was varied from 40 g/L to 80 g/L. All chemicals employed were of analytical grade. One mL of inoculum (1.0 x 10^7 spores/L) was transferred to 250 mL Erlenmeyer flasks containing 50 mL of the desired medium. The flasks were incubated at 25ºC on a rotary shaker at 120 rpm. The mycelia were harvested by filtration through a silkscreen nylon filter. The biomass was lyophilized for 48 hours and kept under vacuum until constant weight. The supernatant was transferred to a penicillin flask for determination of pH, protein and protease activity. The pH was measured in the culture supernatant during cultivation by using a glass-electrode pH meter.

Analytical procedures

Protease activity: The enzyme assay was based on the procedure described by Leighton *et al.* (16). Azoalbumin (Sigma, St. Louis, Mo) was dissolved as a 1% (w/v) solution in 0.1M acetate buffer, 1.0 mM $CaCl_2$, pH 4.5. The solution was stored at -20ºC. A 0.4 mL reaction mixture contained 250 µL of azoalbumin solution and 150 µL supernatant fraction. The reaction was terminated by the addition of 1.2 mL of 10% (w/v) trichloroacetic acid. The reaction tubes were cooled at 0ºC for 15 min and centrifuged to remove the precipitated protein. The supernatant (0.8 mL) was mixed with 1.4 mL of 1N NaOH and the absorbance read at 440 nm. Units of proteolytic activity were expressed as mg of azoalbumin hydrolyzed per hour. Specific activity was calculated by the ratio between total protease activity and total protein concentration in 1 mL of sample (U/mg). The extracellular protein content was determined according to the method of Bradford (3). Bovine serum albumin (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was used as the standard.

Glucose concentration: The glucose in the media was estimated by the Glucose Oxidase Method, according to Hanson and Phillips (13).

Statistical procedures

In all experiments, the measurements were carried out with duplicated parallel cultures. The values reported are means ± S.D, calculated as described by Snedecor and Cochran (24).

RESULTS AND DISCUSSION

The results obtained in this work revealed the ability of *M. circinelloides* to produce extracellular proteases. Fig. 1 (A, B, and C) shows the curves of growth, medium pH, D-glucose concentration and specific protease activity, produced in the culture medium for different D-glucose concentrations. Although the cultivation media had different substrate concentrations, the growth curves, in a general mode, showed a similar profile. It could be observed that the sporic germination time reached almost 16 hours of cultivation. Probably, this improductive period could be avoided by the use of a more concentrate inoculum. This strategy must be considered for large scale applications.

Growth of the mycelia increased rapidly in the early stages and an increase on biomass production occurred when D-glucose concentration was altered in the media cultivation. For the medium containing D-glucose in a concentration of 80 g/L,
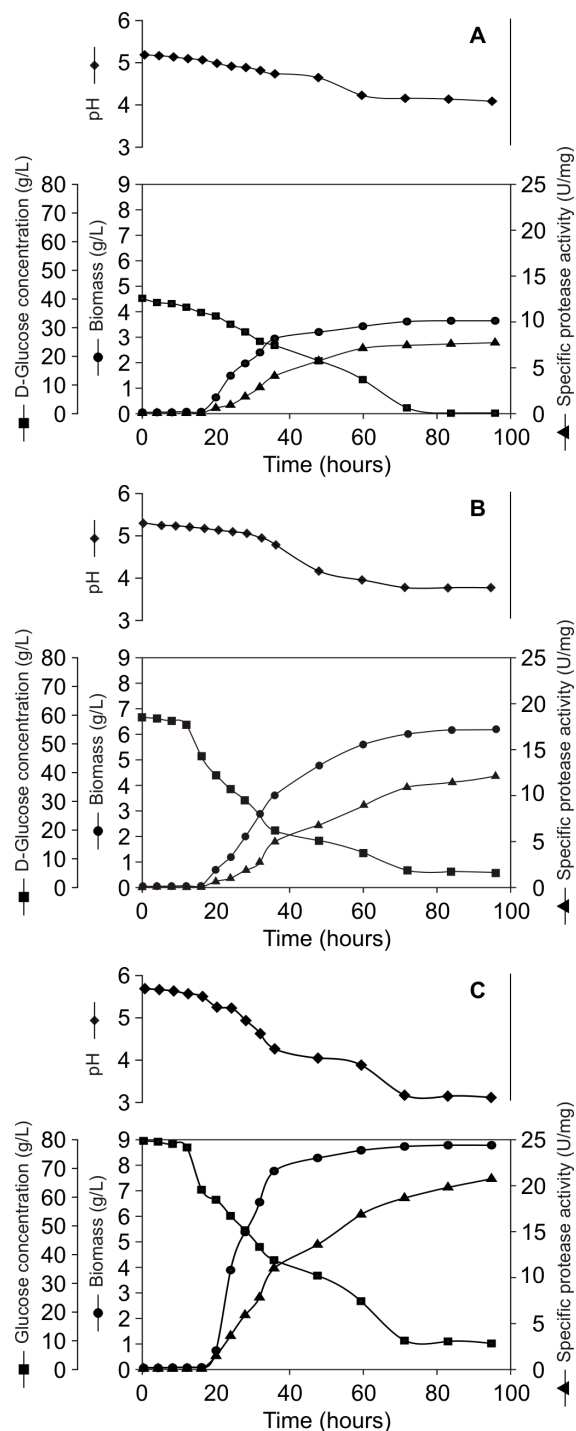
Figure 1. Extracellular protease production by *Mucor circinelloides* IFM 40507 in culture medium containing: 40 (**A**), 60 (**B**) and 80g D-glucose/L (**C**). (−●−) Biomass; (−■−) D-glucose concentration; (−▲−) Specific protease activity; (−◆−) pH . The values represent averages of duplicates experiments, which were not significantly different (*p* > 0.05) from one another (biomass, pH and specific protease activity).

the mycelial mass concentration was higher than those obtained in media containing 40 g/L and 60 g/L D-glucose as shown in Fig. 1. In the media containing higher D-glucose concentrations (60 g/L and 80 g/L), it was observed that the substrate was not totally consumed, in opposition to the medium with 40 g/L D-glucose, where no remaining substrate was detected at the end of cultivation (96 hours of growth).

The pH decreased in the period of enzyme synthesis, and stayed relatively constant up to the end of fermentation. The low pH values observed at the end of cultivation were, probably, consequence of metabolites accumulation resulting from D-glucose degradation as low pH values (3.95 and 3.15) were observed in media containing higher D-glucose concentrations of 60 g/L and 80 g/L, respectively.

To investigate the efficiency of filamentous fungus *Mucor circinelloides* on protease production, for three experimental conditions, the process response factors were calculated and are described in Table 1.

The substrate utilized allowed the microorganism to reach the exponential growth phase, where the micelial specific growth rates (μ_x) were similar for the three glucose concentrations studied.

An important factor is the repression of product formation either by other metabolites or by the main product itself. Kinetically speaking, this behavior is found when the rate of product formation decreases when the substrate concentrations increase (7). In our case, the biomass production increased with glucose concentration as shown in Fig.1 (A, B and C), although the yield factor for product on substrate consumed ($Y_{P/S}$) decreased with glucose concentration (Table 1).

The results obtained here suggest a possible glucose regulation by catabolic repression over the enzymatic production, which can be attributed to the presence of high substrate concentration (80 g/L). This observation is supported by the fact that the yield factor for product on substrate consumed ($Y_{P/S}$) decreased while the yield factor for cell on substrate ($Y_{X/S}$) increased. According to our observations, Dekleva *et al.* (6) described the possibility of these proteases to be repressed by D-glucose remaining in the medium. In contrast, recent investigations showed that protease from *Streptomyces ambofaciens* was detected only after glucose depletion (2).

It has been frequently described that in a defined medium, a protein source must be present for the enzyme to be produced. However, it has been also noticed that the lack of glucose results in a dramatic decrease in enzyme production. Glucose has been reported to suppress protease production (22,25), but in the present study it was found to be a relatively good substrate for enzyme production, specially when used at low concentrations (40 g/L). Other workers have also reported better protease production in the presence of glucose as a substrate (11).

Phadatare *et al.* (19) evaluated various sugars such as glucose, fructose, lactose, maltose, sucrose, xylose, and sugar alcohols, glycerol, mannitol, and sorbitol (1-5%, w/v) for their effect on

Table 1. Process response factors of extracellular proteases production from *Mucor circinelloides*.

Substrate concentration (g/L)	$Y_{X/S}$ (g/g) Yield factor for biomass on substrate consumed	$Y_{P/S}$ (U/g) Yield factor for product on substrate consumed	$Y_{P/X}$ (U/g) Yield factor for product on biomass	Q_P (U/L.h) Volumetric productivity	μx (h^{-1}) Maximum specific growth rate
40	0.09	0.83	8.93	4.57	0.09
60	0.11	0.22	1.92	4.36	0.10
80	0.12	0.14	1.11	3.59	0.13

Values represent the mean of duplicate determinations of duplicate experiments. The standard deviations were less than 1.8%.

enzyme production . The results obtained revealed that sucrose (3% w/v) gave the maximum protease activity of 0.5 U/mL. Yang *et al*. (26) studied the effect of carbon sources on the production of protease by *Bacillus subtillis* growing in shrimp and crab shell powder medium containing one of the additional carbon sources: glucose, lactose, carboxymethyl cellulose, D(-)arabinose, D(+)xylose, and rice bran. They found that protease production was greatly enhanced by the addition of lactose or arabinose into the medium and that 1% arabinose was the most effective substrate and concentration for protease production.

Another important point would be the energetic stock proportionate to the microorganism by glucose and/or other substrates which can favor and regulates the extracellular protease synthesis by *M. circinelloides*. The evidence that extracellular protease regulation may be dependent on the energetic stock of the cell was described in *Pseudomonas aeruginosa* (28) and *Aspergillus nidulans* (18).

Aleksieva and Peeva (1) found that protease production was growth associated when using the fungus *Humicola lutea* cultivated in a medium containing glucose at low concentration (30 g/L). Similarly, the kinetic pattern found in this work describes a growth associated to the product formation for 40 g/L of substrate since the product is produced simultaneously to microbial growth (Fig. 1A). However, for higher substrate concentrations, the growth is typically semi-associated since product formation takes place during the growth and stationary phases (Fig. 1B and 1C), as described in the literature (23,27).

Considering the interest of this study in evaluate protease production from *Mucor circinelloides*, it is not advantageous, for economic application, utilize high glucose concentrations, since the best enzymatic yield occurred for the lowest substrate concentration, as showed either by the $Y_{P/X}$ and $Y_{P/S}$, and by the volumetric production factor (Q_P) which was basically the same for three substrate concentrations studied (Table 1).

Various microorganisms and cultivation media have been studied for protease production (12). The results presented here show that *M. circinelloides* is a promising alternative source of protease, exhibiting volumetric production factor of approximately 4.6 U/L.h.

From the different data accumulated in this research, there was a strong indication that the kinetics factors reported

here could serve as a starting point for an experimental optimization of *M. circinelloides* growth for large-scale protease production. Further studies are in progress to investigate the physiological significance of the protease in relation to the microorganism.

RESUMO

Produção de proteases extracelulares por *Mucor circinelloides* utilizando D-glicose como substrato

Recentemente, algumas espécies da ordem Mucorales têm sido reportadas como produtoras de proteases. Neste trabalho a produção de proteases extracelulares foi estudada em uma amostra de *Mucor circinelloides* utilizando D-glicose, em diferentes concentrações (40, 60 e 80 g/L), como substrato. A fermentação foi acompanhada com determinação de biomassa e pH, assim como pela determinação da atividade proteásica. Embora a produção de biomassa tenha alcançado melhores rendimentos na presença de 80 g/L de glicose, a produtividade enzimática aumentou quando a concentração do substrato foi reduzida para 40 g/L. Os fatores de rendimento de produção enzimática por biomassa, assim como o rendimento de glicose consumida em atividade proteásica foram superiores quando o cultivo do microrganismo ocorreu em meio contendo 40 g/L de D-glicose. As variáveis de resposta do bioprocesso sugerem que a linhagem estudada possa ser utilizada como microrganismo agente para a produção de protease.

Palavras-chave: proteases extracelulares, D-glicose, substrato, *Mucor circinelloides*.

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